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13. ABSTRACT (<i>Maximum 200 Words</i>) Purpose: The main objectives of the proposal are to measure the molecular changes induced by androgen ablation and radiotherapy, to relate these changes to the supra-additive apoptotic response of androgen ablation plus radiation, and to use these data to develop a gene therapy strategy. Scope: Prostate cancer is the most common cancer in men, yet our understanding of the interaction of androgen ablation and radiation at the molecular level is severely lacking. The proposed project will define the role of the key proteins in the apoptotic pathway, p53, bax, and bcl-2. Major Findings: Considerable progress has been made on two fronts: first, characterization of pretreatment biomarker levels in human prostate cancers and second, the development of a gene therapy strategy using adenoviral-p53 and adenoviral E2F. In the latter studies, these vectors have been found to sensitize both LNCaP and PC3 cells to radiation, and preliminary data with Ad5-p53 suggest an added benefit when androgen ablation is added. Some delays have been encountered in measurement of biomarker level changes in LNCaP cells cultured in androgen deprived medium. The problems with the system have been resolved and this analysis is now underway. The immediate significance of these studies is that two gene therapy strategies have been developed and a clinical trial written to test the feasibility, morbidity and efficacy of this approach. The significance of the androgen ablation mechanism/biomarker studies will be manifest at the end of next year.			
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TABLE OF CONTENTS

<u>Section</u>	<u>Page</u>
Front Cover.....	1
SF 298.....	2
Foreword	3
Table of Contents.....	4
Introduction.....	5
Body.....	5
Research Accomplishments.....	14
Reportable Outcomes.....	15
Conclusions.....	15
References.....	16
Appendices.....	18

INTRODUCTION

Hormone deprivation has been shown to be effective at reducing prostate growth, causing prostate cancer cell death and more recently in sensitizing prostate cancer cells to radiation. Despite knowledge of the former two effects since the 1940's, very little is understood about the molecular mechanisms involved. The focus of the research here is to dissect the molecular mechanisms mainly through the use of human tumor models cultured in vitro. Three key proteins of the apoptotic pathway have been targeted, p53, bax, and bcl-2, because of data from our group that under certain conditions androgen ablation (AA) when combined with radiation (RT) results in supra-additive cell killing in the form of apoptosis (Lim Joon et al, 1997; Pollack et al, Submitted). Our data have shown that when androgen ablation is started 3 – 14 days prior to single fraction 2 – 8 Gy irradiation, a 5 – 10 fold increase in apoptosis is observed over AA only and RT only controls. These *in vivo* data were obtained using the Dunning R3327-G line. Supra-additive apoptosis was found to be sequence-specific and time-limited, suggesting that the temporal alterations in key molecular proteins in the apoptotic pathway were occurring immediately following AA and over the ensuing two weeks. After 2 weeks, supra-additivity was lost. The results indicate that the molecular alterations from AA that initially predisposed to apoptosis from irradiation were lost after two weeks, leading to the re-emergence of resistance to irradiation. More recent data have demonstrated that supra-additive apoptosis is not sustained with multiple radiation fractions (Pollack et al, Submitted; see Appendix 1). Thus, the addition of radiation to AA further changes the molecular environment, such that apoptosis on repeated fractions is not evidenced, even during the two week period in which single fraction doses cause this effect. Defining the molecular changes promoted by AA and then by irradiation in multiple fractions is the emphasis of the proposed work. The goal was to then develop a gene therapy approach to enhance apoptosis and consequently overall tumor cell death based on manipulating proteins in this pathway.

BODY

Task 1. To measure alterations in the expression of p53, bax, and bcl-2 proteins induced by androgen ablation alone.

- a. Western blots, immunohistochemistry, and TUNEL assay of cultured LNCaP cells to determine the changes in p53, bax, and bcl-2 expression following androgen depletion and the relationship of these changes to the induction of apoptosis (months 1-6).
- b. Immunohistochemistry of pretreatment levels of p53, bax, and bcl-2 in formalin-fixed paraffin-embedded human prostate tissue samples from patients that have undergone radiotherapy (months 6-12)
- c. Immunohistochemistry of pretreatment and post-treatment levels of p53, bax, and bcl-2 in formalin-fixed paraffin-embedded human prostate tissue samples from patients that have undergone radical prostatectomy after androgen ablation (months 12-18).

Task 1, Part a, Western blots, immunohistochemistry, and TUNEL assay of cultured LNCaP cells to determine the changes in p53, bax, and bcl-2 expression following androgen depletion and the relationship of these changes to the induction of apoptosis:

The measurement of alterations in the expression of p53, bax, and bcl-2 in response to androgen ablation in vitro was predicated on having an in vitro system that correlated with the in vivo AA + RT data (Pollack et al, 1997; Lim Joon et al, 1997; Pollack et al, Submitted). At the time the grant was submitted, supra-additive apoptosis had been observed in preliminary studies using LNCaP cells cultured in charcoal-stripped medium for 2 d and then irradiating with a single fraction of 2 – 8 Gy. However, for most of the first year, we had problems consistently eliciting supra-additive apoptosis in this system; in some experiments it was clearly seen, while in others it was absent. As a consequence, we contemplated abandoning LNCaP cells or possibly going back to an in vivo system. The problem with in vivo experiments using human tumors grown in nude mice is that mouse elements (stroma, vessels) infiltrate the tumors and complicate molecular studies. We therefore concentrated effort on establishing consistency in the system, which was accomplished over the last few months.

The in vitro tumor cell growth response to AA was assessed by culturing the cells in charcoal-stripped medium, unstripped medium, or charcoal stripped medium plus R1881 (androgen replacement). Figure 1 is a representative experiment of three, showing that cell growth is inhibited in charcoal-stripped medium and that partial reversal of this effect is accomplished by culturing the cells in androgen supplemented charcoal-stripped medium using 1×10^{-10} M R1881.

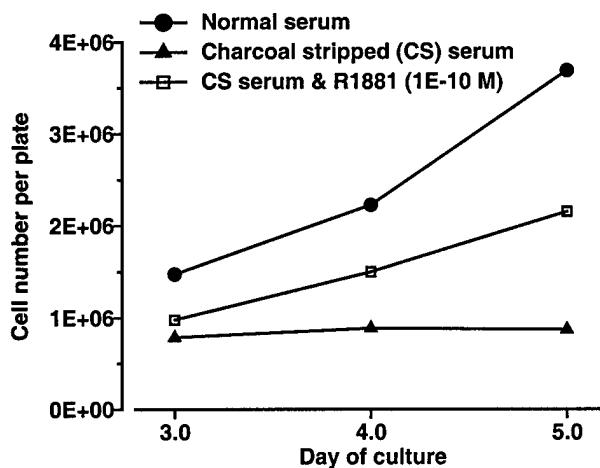


Figure 1. Cell growth response of LNCaP cells cultured in vitro to androgen ablation. Charcoal stripped serum was used deplete the medium of androgen.

Supra-additive apoptosis in response to AA+RT in vitro in LNCaP cells was then documented using the TUNEL assay. In Table 1, LNCaP cells were cultured in normal serum, charcoal stripped serum, or charcoal stripped serum plus androgen using R1881 at 1×10^{-10} M. The cells were exposed to 0, 2, or 8 Gy irradiation 3 days after exposure to androgen deprivation. Although some inter-experiment variability was seen, there clearly was supra-additivity in the apoptotic response of LNCaP cells to single fraction irradiation at 2 and 8 Gy. Replacement of androgen using R1881, caused the suppression of apoptosis to near the control levels with

normal serum. The in vitro model system that has been developed will be invaluable in delineating the molecular mechanisms involved.

Table 1. In vitro apoptosis response of LNCaP cells to androgen ablation plus radiation.

Serum Group	Radiation (Gy)	AI		
		Exp 1	Exp 2	Exp 3
Normal	0	0	0	0
Normal	2	0	0.2	0
Normal	8	0.4	0.1	0.4
Charcoal stripped	0	14.3	5.1	2.0
Charcoal stripped	2	14.2	11.8	5.1
Charcoal stripped	8	22.4	18.5	6.25
Charcoal Stripped + R1881	0	0.2	3.1	1.0
Charcoal Stripped + R1881	2	0.7	7.0	0.1
Charcoal Stripped + R1881	8	1.0	6.35	0.5

A minimum of 2000 tumor cells were counted to determine the percentage of TUNEL positive cells (AI). The samples were counted by two individuals and the average of the counts are shown. One person was blinded to the groups and the counts between the two individuals was similar.

Further characterization of the in vitro system LNCaP system is still needed. However, the intent is to move directly to the molecular studies using Western blots while characterization continues.

Task 1, Part b, Immunohistochemistry of pretreatment levels of p53, bax, and bcl-2 in formalin-fixed paraffin-embedded human prostate tissue samples from patients that have undergone radiotherapy:

The goal of this task was to assess the pretreatment levels and prognostic value of p53, bax, and bcl-2. These human data will then be compared with levels seen after androgen ablation (Task 1, Part c). The human tissue findings on the effects of AA on molecular marker levels would then be related to the changes seen in the LNCaP model. Over 700 letters were sent in an attempt to retrieve pretreatment tissue from patients that underwent external beam radiotherapy at M.D. Anderson Cancer Center between 1987 and 1993. All patients had a pretreatment PSA and none were treated with neoadjuvant or adjuvant AA. Unstained, formalin-fixed, paraffin embedded tissue was obtained in 150 patients and, of these, 106 had tissue suitable for analysis. From these data, one paper is in press and two in preparation.

A major component of these studies was to test the independence of the molecular markers in predicting outcome, when analyzed with the known prognostic factors of pretreatment PSA, Gleason score, and clinical stage. Since DNA-ploidy and Ki-67 labelling index have also been repeatedly shown to be associated with patient outcome, these markers were also analyzed. In one study (Khoo et al, In Press, see Appendix 2), all of these pretreatment

features were measured with the objective of evaluating the relationship between DNA-ploidy and Ki-67 labelling index. DNA ploidy was quantified by flow cytometry (Gauwitz et al, 1993; Pollack et al, 1994) and the Ki-67 labelling index (Ki67-LI) was quantified immunohistochemically using the MIB1 antibody (Cattoretti et al, 1992). The cohort used in this report consisted of 42 patients that underwent transurethral resection of the prostate prior to treatment, and therefore, had sufficient material for both assays. The results showed that Ki67-LI was a strong correlate of freedom from a rising PSA in patients treated with radiotherapy. Ki67-LI appeared to superior to DNA-ploidy, although the patient numbers were too small for a valid multivariate analysis. Patients with a Ki67-LI of >3.5% had a poor prognosis (Fig 2).

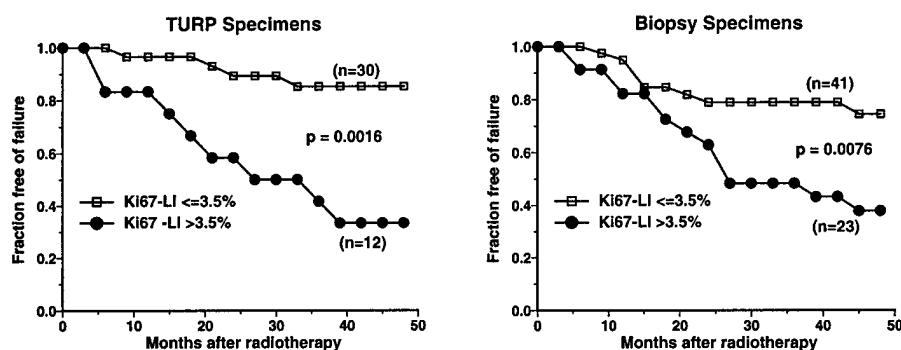


Figure 2. Relationship of Ki67-LI to actuarial freedom from biochemical failure in patients analyzed from TURP specimens (left) and those with biopsy specimens (right).

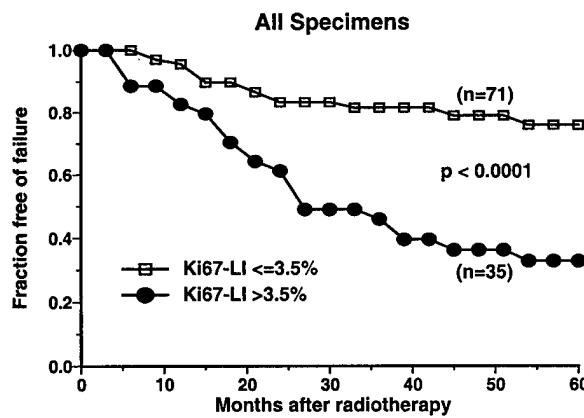


Figure 3. Relationship of Ki67-LI to actuarial freedom from biochemical failure in the entire cohort of 106 patients.

A second analysis using the entire cohort of 106 patients is nearly complete. The value of Ki67-LI in predicting patient outcome was determined, using the cut-point of 3.5% defined in

the above study. There were a total of 106 patients, 42 that had TURP specimens that constituted the initial cohort in whom the cut-point was determined, and 64 that had biopsy specimens that constituted the test cohort. Figure 2 shows that using this same cut-point in the test cohort correlated with freedom from biochemical failure; those with a Ki67-LI $>3.5\%$ had a significantly worse prognosis than those with a Ki67-LI $\leq 3.5\%$. Figure 3 illustrates the univariate freedom from biochemical failure results for the entire cohort. Multivariate analysis using Cox proportional hazards confirmed Ki67-LI to be significantly associated with this endpoint, independent of pretreatment PSA, Gleason score, and stage.

The next markers analyzed were bcl-2, bax, and bclx. Immunohistochemical staining was performed and if any tumor cells were stained, the sample was considered positive for the marker. Of the 106 samples that were used for the ki67 analysis, there 77 that had adequate tumor and staining for bcl-2. Twelve of these samples stained positive (16%) and the results show that these patients has significantly higher actuarial biochemical failure rates than patients with bcl-2 negative samples (Figure 4). A preliminary analysis of bclx in 51 patients has thus far not yielded any statistically significant difference (Figure 4), despite the finding of a nearly even distribution of positives (56%) and negatives (44%). Preliminary results of the staining for bax were available on 49 patients and only two stained positively. Thus, the only promising marker , in terms of an association with patient outcome, was bcl-2, and the results with this marker were relatively weak because on 16% demonstrated altered expression. Nonetheless, multivariate analysis using Cox proportional hazards revealed that bcl-2 was an independent correlate of freedom from biochemical failure, along with pretreatment PSA, Gleason score, stage, and Ki67-LI.

The staining for p53 was placed at the bottom of the list, because our prior results have shown that in the relatively early stage patients that comprise this cohort, abnormal p53 expression, as determined by immunohistochemical staining is low (Hall et al, 1995). Staining of the samples for p53 is in progress.

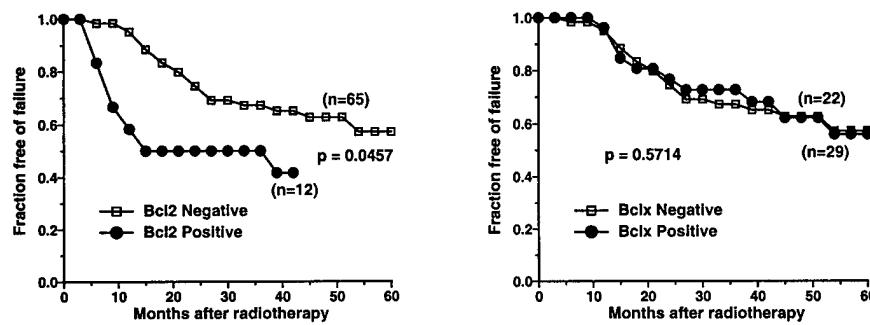


Figure 4. Immunohistochemical staining of bcl-2 (left) and bclx (right) of prostate tumor samples from patients treated with radiotherapy at M.D. Anderson Cancer Center.

Cox proportional hazards regression showed that bcl-2 staining was a significantly independent correlate of freedom from biochemical failure, along with pretreatment PSA, Gleason score, stage, and Ki67-LI. The next step in the investigation of the expression of these molecular markers is to determine the alterations that are evidenced with androgen ablation (Task 1, Part c).

Task 1 Timelines

Part a: Year 2, Months 1 – 6:

Perform Western blots for p53, bax, and bcl-2 on LNCaP cells cultured for 1, 2, 3, 4, and 5 days in normal serum, charcoal stripped serum, or charcoal stripped serum plus R1881.

Determine the apoptotic response of cultured LNCaP cells to multiple radiation fractions. These studies will be done to see if this system is similar to the R3327-G rat prostate model in which apoptosis was much lower for the second fraction (Pollack et al, Submitted; see Appendix 1). LNCaP cells will be cultured for 3 days in either normal serum, charcoal stripped serum, or charcoal stripped serum plus R1881. Then the cells will be irradiated with 2 daily fractions of 2 or 8 Gy and apoptosis measured 24 hr after the second fraction.

Effect of AA+RT on clonogenic survival. Several experiments have already been performed and the results have been inconsistent. Some experiments have shown that androgen deprivation via culture in charcoal stripped serum causes reduced clonogenic survival, but other experiments have not been confirmatory. The documentation of a survival effect is important and the plan is to try to improve reproducibility in repeated experiments.

Part b, Year 2, Months 1 – 6:

Complete immunohistochemical staining of p53, bclx, and bax.

Part c, Year 2, Months 1 – 6:

Begin and complete immunohistochemical staining of p53, bclx, and bax in tumor tissue specimens from patients that have been treated with androgen ablation prior to radical prostatectomy.

Task 2. To assess whether the mechanism of the supra-additive apoptotic response to androgen ablation plus single fraction radiation and the lack of this response with high single fraction or fractionated doses, is related to changes in p53, bax, or bcl-2 expression.

- a. Western blots, immunohistochemistry, and TUNEL assay of cultured LNCaP cells to determine if changes in p53, bax, or bcl-2 expression correlate with the maximal supra-additive apoptotic response from single dose radiation (2 or 8 Gy) administered at different times after androgen depletion (months 6-12).
- b. Western blots, immunohistochemistry, and TUNEL assay of cultured LNCaP cells to assess the relationship of changes in p53, bax, or bcl-2 levels to apoptosis when high doses (16 - 32 Gy) are given at approximately 72 hr (determined from above experiment) after androgen ablation (months 12-18).
- c. Determine the dose-response relationship of cell death by apoptosis to clonogenic cell survival in cultured LNCaP cells treated with androgen ablation and single doses of radiation (1-32 Gy). (months 12-18)

- d. Western blots, immunohistochemistry, and TUNEL assay of cultured LNCaP cells to assess the relationship of changes in p53, bax, or bcl-2 levels to apoptosis when fractionated daily doses (2 or 8 Gy) are given at approximately 72 hr (determined from above experiment) after androgen depletion (months 18-24).
- e. Determine the dose-response relationship of cell death by apoptosis to clonogenic cell survival in cultured LNCaP cells treated with androgen ablation and daily fractionated doses of radiation (2 or 8 Gy). (months 18-24)

With the exception of Part a, Task 2 was to be performed in the second year. For Part a, the TUNEL experiments have been performed, as described above. The immunohistochemistry staining and westerns of p53, bax, and bcl-2 will be done in the first 6 months of Year 2, with the other Parts of Task 2. Completion of Task 2 is anticipated by the end of this year or early next year.

Task 3. To optimally integrate androgen ablation and radiation based on the molecular marker data obtained and to further enhance apoptosis to this regimen using gene therapy

- a. Optimize the length of administration of androgen ablation, the fractionation of radiation and the sequencing of androgen ablation and radiation to maximize the molecular conditions that promote apoptosis and a consequent decrease in clonogenic cell survival (months 24-30).
- b. Gene therapy, probably using an adenoviral p53 or bax vector, to sustain the intracellular conditions identified as being ideal for supra-additive apoptosis with androgen depletion plus radiation (months 24-30)

The goal of Task 3 was to take the information from Tasks 1 and 2 and devise a gene therapy treatment strategy. However, the problems encountered with the in vitro LNCaP model (which have now been resolved), combined with the preliminary favorable results with adenoviral gene therapy, led to the investigation of this Task ahead of schedule. The results have been very favorable and the first manuscript on the in vitro studies using an adenoviral-p53 (Ad5-p53) vector is nearly complete (see Appendix). Two vectors have been characterized, Ad5-p53 and Ad5-E2F.

Ad5-p53 Studies: Two assays were used to characterize the effects of Ad5-p53 on prostate cancers cells in vitro and whether transgene p53 expression sensitizes such cells to radiation: clonogenic survival and apoptosis by TUNEL. The LNCaP model, which is p53^{wildtype}, and the PC3 model, which is p53^{null}, were used. The data showed that Ad5-p53 sensitized both LNCaP and PC3 cells in vitro (see Appendix). These in vitro results have been extended to in vivo systems, in which LNCaP cells were grown orthotopically in the prostates, and PC3 cells in the legs, of nude mice.

PC3 cells (2×10^6) were injected into the hind legs of nude mice. Treatment with Ad5-p53 was initiated at a tumor volume of 200 mm^3 . Three intratumoral injections (days 1, 4, and 7) were given with 3×10^8 plaque forming units, followed by 5 Gy pelvic irradiation in one fraction using a Cobalt-60 source. Tumor volume measurements were obtained every 2 days. LNCaP cells (2×10^6 in 24 ul) were injected orthotopically into the prostates of nude mice and tumor weight approximated using serum PSA obtained from weekly tail vein bleedings. There is a linear

relationship between tumor weight and serum PSA; linear regression results revealed that tumor weights of 7, 10, and 20 mg correlated with PSAs of 1.4, 3.0, and 8.5 ng/ml. The target PSA for the studies was 5 ng/ml. The animals were then anesthetized, the prostate surgically exposed, and 4.5×10^8 pfu injected in 24 ul. The intraprostatic injections were done twice (days 1 and 2), and 5 Gy pelvic irradiation was administered 24 hr later (day 3).

The PC3 tumor volume growth curves (Figure 5) were log-transformed, and fitted using linear regression. The times (in days) for the tumors to reach 500 mm^3 were calculated as 10.7 ± 0.7 for the saline control (no virus), 9.8 ± 2.1 for Ad5-pA, 15.6 ± 1.6 for Ad5-p53, 14.6 ± 1.5 RT (5 Gy), 14.6 ± 1.5 for Ad5-pA plus RT, and 31.3 ± 5.3 for Ad5-p53 plus RT. The latter group times were significantly different at $p < 0.05$ (one way ANOVA, Scheffe test) from all of the other groups. The absolute delay in tumor growth to 500 mm^3 relative to the saline control was used to calculate the enhancement factor [Abs delay(Ad5-p53+RT - Ad5-p53)/RT], which was 4.0; this value is consistent with supra-additivity. The pA controls were not included in the calculation because no significant delays were seen.

Ad5-p53 Treatment of PC3 in vivo

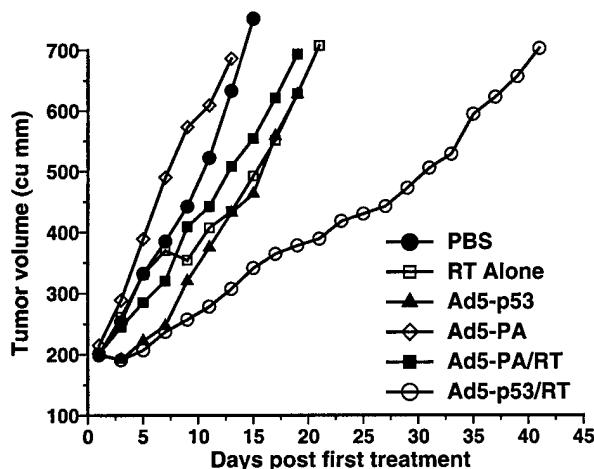


Figure 5. Ad5-p53 treatment of PC3 tumors grown in the legs of nude mice.

LNCaP tumor growth was determined via weekly serum PSA measurements, and a serum PSA of >1 ng/ml six weeks after treatment was considered evidence of regrowth. There were five animals per group. The number of animals with evidence of regrowth were 5 for the saline control, 5 for Ad5-pA, 4 for Ad5-p53, 4 for RT alone (5 Gy), 5 for Ad5-pA + RT, and 1 for Ad5-p53 + RT. These results are also consistent with a supra-additive inhibition of tumor growth.

In conclusion, for these in vivo gene therapy experiments tumor growth was inhibited supra-additively when null-p53 and wild type-p53 prostate tumors were treated with Ad5-p53 and 5 Gy radiation. These studies have led to the initiation of a clinical trial (see Appendix 3) that has been submitted for consideration of funding as part of another Department of Defense grant.

Another series of experiments that have been initiated revolve around the hypothesis that Ad5-p53 gene therapy may sensitize androgen sensitive cells to androgen deprivation. Preliminary results (Table 2) have thus far not shown this to be the case for p53^{wildtype} LNCaP cells grown in the prostate of nude mice. Although more animal numbers are needed, there is a suggestion of increased biochemical control when p53, androgen ablation, and radiation are combined.

Table 2. Effect of Ad5-p53, Castration and 5 Gy Radiation on biochemical control for LNCaP cells grown orthotopically in the prostates of nude mice.

Group	Biochemical Control at 6 weeks	
	No Radiation	5 Gy Radiation
No Treatment	0/5(0%)	2/10(20%)
Ad5-pA	0/10(0%)	0/5(0%)
Ad5-p53	2/10(20%)	4/5(80%)
Castration	6/10(60%)	7/10(70%)
Castration & Ad5-p53	5/9(56%)	9/10(90%)

Treatment was initiated when tumors had an average pretreatment PSA of 5 ng/ml. Biochemical control assessed at 6 weeks following treatment and was defined as a PSA of ≤ 1 ng/ml.

Ad5-E2F Studies: The pathway controlling the progression of cells from quiescence through G1, and into S phase involves the activation of G1 cyclin-dependent kinases, inactivation of Rb and related proteins, and accumulation of E2F transcription factor activity. Like other signal transduction activities, E2F consists of a family of related proteins that include distinct E2F members and at least two heterodimer partners, DP1 and DP2. The first member of this family E2F-1 has been suggested to play a key role in the regulation of cell-dependent gene expression and apoptosis. Phosphorylation of Rb results in the activation of E2F-1, which is thought to drive the G1 to S phase transition. In addition to cell cycle activation, E2F-1 overexpression promotes S-phase entry followed by p53-dependent apoptosis (Qin et al, 1994; Kowalik et al 1995). Apoptosis activity of deregulated E2F-1 seems to occur by the activation of p19 as a consequence of p53 stabilization (DeGregori et al, 1997). The absence of phosphorylation and inactivation of E2F-1 DNA binding function by cyclin A kinase induces S-phase arrest/delay followed by apoptosis (Krek et al, 1995). Apoptosis induction via E2F-1 may occur through p53 independent mechanisms; E2F-1 mediated apoptosis has been observed in mutant p53 ovarian and breast carcinoma cell lines (Hunt et al, 1997).

The modulation of radiation response through biologic therapy is only beginning to be studied and the potential is tremendous. The E2F-1 vector offers a means of enhancing radiation induced apoptosis and our preliminary results described below in p53^{wild-type} LNCaP cells suggest that this effect is pronounced. It has also been demonstrated previously that exposure of p53^{wild-type} E1A/ras-transformed cells to ionizing radiation results in a decrease in cell viability even at low doses, as compared to p53^{null} cells (Lowe et al, 1993). Although deregulated expression of E2F-1 displayed little effect on the clonogenic growth of p53^{null} cells, the sensitivity of cells to radiation after transgene E2F-1 overexpression is not known.

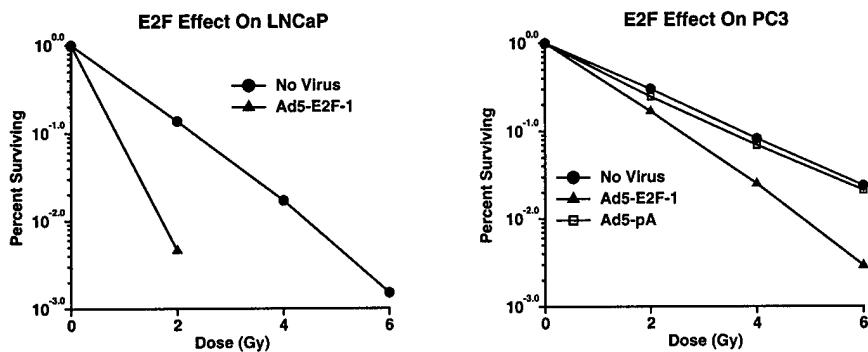


Figure 6. Clonogenic survival of LNCaP (left) and PC3 (right) cells to Ad5-E2F with and without radiation. The curves were corrected for differences in plating efficiency when no radiation was given.

The effect of E2F-1 transgene expression with and without radiation was examined in LNCaP cells (Salem et al, Abstract In Press). An Ad5-E2F-1 vector with a CMV promoter was used and Ad5-CMV-PolyA (Ad5-pA) was used as a control. The viral solutions were used at a multiplicity of infection of 10 and 25. The cells were irradiated with 2, 4, and 6 Gy 24hr after transfection. The plating efficiency dropped from 41.3% to 1.9% using Ad5-E2F-1 without irradiation. The LNCaP cell line was found exquisitely sensitive to radiation in the presence of E2F-1 overexpression. The combination at 2 Gy dramatically reduced clonogenicity in LNCaP by over 30 fold, as compared to 2Gy in the absence of E2F-1 (Figure 6). The results in Figure 6 also show that Ad5-E2F sensitized p53^{null} PC3 cells to radiation, but, to a reduced degree as compared to p53^{wildtype} LNCaP cells. These results suggest that the Ad5-p53 and Ad5-E2F gene therapy vectors might be complimentary.

KEY RESEARCH ACCOMPLISHMENTS

- More completely characterized an in vitro system using LNCaP cells that documents supra-additive apoptosis when androgen deprivation and single fraction radiation are sequenced under certain conditions.
- Have shown that immunohistochemical staining results for Ki-67/MIB-1 and bcl-2 are independent correlates of freedom from biochemical failure in human prostate cancer patients treated with radiotherapy. Preliminarily, pretreatment Bax and bclx expression were not significant.
- Gene therapy with Ad5-p53 sensitized both p53 wildtype LNCaP and p53 null PC3 cells to radiation by clonogenic survival and apoptosis. These promising results were confirmed in *in vivo* and have led to a protocol in prostate cancer patients (see Appendix).
- Gene therapy with Ad5-E2F sensitized both p53^{wildtype} LNCaP and p53^{null} PC3 cells to radiation by clonogenic survival and apoptosis. The sensitization pattern was complimentary

to that of Ad5-p53 in that maximal radiosensitization for Ad5-p53 and Ad5-E2F was observed for PC3 and LNCaP cells, respectively.

REPORTABLE OUTCOMES

Manuscripts

- 1) Khoo VS, Pollack A, Cowen D, Lim Joon D, Patel N, Terry NHA, Zagars GK, von Eschenbach AC, Meistrich ML, Troncoso P. Relationship of Ki-67 labeling index to DNA-ploidy, S-phase fraction, and outcome in prostate cancer treated with radiotherapy. *Prostate* 1999 (In Press).
- 2) Kelly J, Pollack A, Zagars GK. Serum testosterone is not a correlate of prostate cancer lymph node involvement, but does predict biochemical failure for lymph-node-positive disease. *Urol. Oncol.* 1999 (In Press).
- 3) Pollack A, Ashoori F, Sikes C, Lim Joon D, von Eschenbach AC, Zagars GK, Meistrich ML: The early supra-additive apoptotic response of R3327-G prostate tumors to androgen ablation plus radiation is not sustained with multiple fractions. *Int J radiat Oncol Biol Phys* (Submitted).

Abstracts

- 1) Cowen D, Salem N, Ashoori F, Meyn R, Meistrich ML, Pollack A. Prostate cancer radiosensitization in vivo with adenovirus-mediated p53 gene therapy. *Int. J. Radiat. Oncol. Biol. Phys.*(In press). *To be presented at the annual ASTRO meeting Oct 31-Nov 4, 1999.
- 2) Salem N. Adenoviral-mediated E2F-1 expression sensitizes prostate cancer cells in vitro to Ionizing radiation. * To be presented at the annual ASTRO meeting Oct 31-Nov 4, 1999.

Protocols

Principal Investigator: A. Pollack

Title: "A phase II randomized study of adenovirus-p53 plus radioactive seed implant versus seed implant alone for PSA relapse after external beam radiotherapy"

Related Grant Applications

Principal Investigator: Christopher Logothetis

Role: Co-investigator (A. Pollack)

Title: "Development and application of novel therapy targeting program in all death in advanced prostate cancer"

CONCLUSIONS

The LNCaP model has proven to be a useful in vitro system for examining the interaction of androgen deprivation plus radiation using apoptosis as an end point. The problems encountered early in the project with reproducibility have been overcome. Task 1 should now be completed relatively quickly. No other in vitro correlate of the supra-additive apoptosis has been

described in the literature; this model provides a unique opportunity to detailed molecular studies under controlled conditions that are not achievable *in vivo*.

Although the human pretreatment biomarker studies were not scheduled to be completed until year two, valuable prognostic information has been gained and papers have been submitted and are in preparation. It appears that Ki-67/MIB-1 is an independent correlate of prostate cancer patient outcome, when treated with radiotherapy. Preliminary data also indicate that bcl-2 is important. We anticipate publication of these data late this year or early next year. The next step is to determine the shifts in the expression of these markers induced by androgen ablation.

The gene therapy studies have proceeded ahead of schedule and have been very successful. The p53 studies have led to a clinical trial that has just been approved by the IRB at MDACC. The E2F studies are intriguing because the results compliment those obtained with p53, suggesting that the two genes may work in concert to radiosensitize a broader range of prostate cancers. The plan is to pursue this avenue and to further establish if adding androgen ablation to this combination is beneficial.

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- ablation plus radiation is not sustained with multiple fractions. Int J radiat Oncol Biol Phys (Submitted).
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**THE EARLY SUPRA-ADDITIVE APOPTOTIC RESPONSE OF R3327-G PROSTATE
TUMORS TO ANDROGEN ABLATION AND RADIATION IS NOT SUSTAINED WITH
MULTIPLE FRACTIONS^{1,2}**

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ABSTRACT

Purpose: The treatment of R3327-G tumor-bearing rats with androgen ablation (AA) via castration results in a supra-additive increase in apoptosis when 2-8 Gy γ -irradiation (RT) is given as a single dose 3-14 d afterwards. We report here the dose-response and effect of multiple fractions on this supra-additive apoptotic response.

Materials & Methods: Dunning R3327-G tumors were grown in the flanks of Copenhagen rats and the experiments were initiated at a tumor volume of 1.0-1.5 cc. Androgen ablation was achieved by castration 3 days prior to γ -irradiation. Apoptosis was measured with a terminal deoxynucleotidyl transferase dUTP-biotin nick end-labelling assay 6 hr after RT, unless otherwise specified.

Results: The dose-response of the supra-additive apoptotic response was assessed by irradiating castrated animals with single doses of 2, 4, 8 or 16 Gy (n=5 per group); tumor cell apoptosis at 6 hr following irradiation was 2.4 ± 0.7 (+SEM), 4.2 ± 0.8 , 6.5 ± 1.4 , and $1.6 \pm 0.3\%$, respectively. The RT only and AA only controls had <1% apoptosis. The effect of fractionated RT on apoptosis was investigated to determine if the supra-additive apoptotic response was sustained with repeated 2-8 Gy fractions. When tumor-bearing animals were treated with repeated daily 2 Gy fractions there was a reduction in the level of the supra-additive apoptotic response. After five 2 Gy fractions at 24 hr intervals, apoptosis in the combined treated tumors was at levels seen in the AA controls. This raised the possibility that more than 24 hr are required for recovery of the high supra-additive apoptotic levels seen after one fraction. When the interfraction interval was extended to 96 hr, there was no significant increase in apoptosis over the additive effect of AA and RT. Although there was a decline in supra-additive apoptosis with repeated fractions, a

dose-response for tumor growth delay was evident for RT alone using 2.5 Gy fractions. Moreover, the combination of AA + fractionated RT resulted in a supra-additive enhancement in tumor growth delay to 5 cc.

Conclusion: The early supra-additive apoptotic response from androgen ablation and single fraction radiation is not seen at high single fraction doses and is not sustained with repeated fractions. Therefore, the classical apoptotic response, that occurs within 24 hr of irradiation, is not likely to be the main mechanism responsible for any clinical benefit seen with this combination.

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INTRODUCTION

Clinical and laboratory data suggest that androgen ablation (AA) plus radiation (RT) results in improved prostate cancer control rates. Three randomized trials have documented a highly significant increase in freedom from biochemical failure for androgen ablation plus radiation over radiation alone (1-3). A survival benefit was seen in two of the trials. The lack of an androgen ablation alone arm in these studies compromises interpretation because a survival benefit has been described for early androgen ablation, as compared to deferred androgen ablation (4). A key question has been whether the combination of AA+RT results in more cell killing than either treatment given individually and, if so, whether apoptosis is the principal mechanism of this interaction.

Laboratory studies using animal and human prostate cancer models have shown that cell killing is enhanced with AA+RT over either treatment given individually using a TCD50 assay (5-7), tumor growth delay (8) and apoptosis (8). Our experiments with the Dunning R3327-G line have revealed that the concern about making cells resistant to RT by driving them into a quiescent state in response to AA is unfounded. Treatment of R3327-G tumor-bearing rats with AA via castration increases the proportion of quiescent cells from 35 to >90%, with the establishment of a new cell kinetic equilibrium by 3 days (9). Administration of 2-8 Gy as a single fraction at the time this equilibrium is established elicits a 5-10 fold supra-additive apoptotic response. This supra-additive cell killing by AA+RT is sequence specific (not seen if the treatments given together) and time-limited (not seen if RT given 3 weeks after AA).

In this report we examine the significance of the supra-additive apoptotic response when multiple RT fractions are administered.

MATERIALS AND METHODS

Tumor Model. All experiments were performed using the R3327-G Dunning rat prostate tumor model in the 23rd-24th in vivo passage (10, 11). Enzyme dispersed and frozen tumor cells from an earlier passage were grown for one passage in vivo prior to enzyme dispersion and injection of 2×10^7 cells into the flanks of experimental Copenhagen rats (250 g). The experiments were begun when the tumors were 1-2 cc.

Treatment. Androgen ablation was accomplished by trans-scrotal bilateral orchiectomy with anesthesia consisting of 40 mg/kg ketamine (Boehringer Animal Health Inc., St. Joseph, MO) and 1.0 mg/kg acepromazine (Fermenta Animal Health Co., Kansas City, MO) injected intramuscularly. The reversal of androgen ablation (androgen restoration) was achieved by subcutaneously implanting silastic capsules containing testosterone in the flank opposite the tumor. The silastic tubing used for the capsules had a 0.198 cm inner diameter and 0.317 cm outer diameter (Dow Corning 602-305, Midland, Michigan) (8). The capsules were filled to a length of 2.0 cm with testosterone (Sigma Chemicals, St. Louis, Missouri) and both ends were sealed with No. 732 silicone sealant (Dow Corning).

A Cobalt-60 source at a source to skin distance of 80 cm and a dose rate of 68.3 cGy/min was used for irradiation. Intramuscular ketamine/acepromazine anesthesia was used to insure immobilization in a specially designed jig. As described previously (8), the tumor was positioned in the radiation field using pegs, while shielding the rest of the body of the rat with 5 cm thick lead blocks. Four rats were positioned at each edge of a square 16 x 16 cm field and

irradiated simultaneously. Dose homogeneity was accomplished by placing 0.5 cm of tissue equivalent material (superflab) over the tumors.

Measurement of Apoptosis. At the conclusion of the treatments, the tumors were removed, fixed in 10% neutral formalin for approximately 24 hr, and embedded in paraffin. The terminal deoxynucleotidyl transferase dUTP-biotin nick end-labelling (TUNEL) assay was performed on tissue previously sectioned onto silane coated slides (8). Hematoxylin and eosin stained sections were also examined.

The TUNEL assay was performed using the ApopTag in situ apoptosis detection kit (Oncor, Gaithersburg, MD). A positive control, consisting of irradiated mouse intestinal crypts, was included with each staining run. Twenty or more random high power fields were examined and a minimum of 2000 tumor cells counted. Necrotic cells and stroma were excluded. The apoptotic index was determined by dividing the number of apoptotic tumor cells by the total number of tumor cells, multiplied by 100.

Measurement of tumor volume. Tumor length, width, and depth were quantified and tumor volume calculated using the formula $L \times W \times D \times 0.5236$. The effect of treatment on tumor volume growth delay was calculated from log-transformed and fitted tumor volume growth curves as described previously (8,9). Briefly, the absolute delay in time for the tumors to reach 5 cc, relative to the intact controls, was determined for AA (AA control), RT (RT Control), and AA+RT. For these experiments AA was reversed after 7 days using testosterone implants as described above. The enhancement factor was calculated from the equation: Abs delay(AA+RT - AA control)/Abs delay(RT control).

RESULTS

The supra-additive apoptotic response that we reported previously (8) was maximal when single fraction RT was applied 3 d after AA and the tumors harvested 6 hr thereafter. Therefore, the dose-response of the supra-additive apoptotic response was assessed by castrating tumor-bearing animals, waiting 3 days, irradiating with single doses of 2, 4, 8 or 16 Gy (n=5 per group), and removing the tumors 6 hours later. The levels of tumor cell apoptosis were 2.4 ± 0.7 (\pm SEM), 4.2 ± 0.8 , 6.5 ± 1.4 , and $1.6 \pm 0.3\%$, respectively (Figure 1). The RT only and AA only controls had <1% apoptosis; Figure 1 also shows the RT only controls. Because of the unexpected drop in apoptosis from 16 Gy, another experiment was performed to test whether the early apoptotic response to 16 Gy peaked at a different time from when lower doses were used. Apoptosis was measured at 3, 6, 9, and 12 hr after RT. Figure 2 shows that the timing of the apoptotic response to 16 Gy was the same as reported previously for lower radiation doses (8); maximal apoptosis was 6 hr after 16 Gy irradiation in both intact and castrate rats. Some inter-experiment variability (Figure 1 versus Figure 2) in the absolute apoptosis levels for tumors treated with 16 Gy is evident. The apoptotic index for the AA+RT group minus that for the intact RT group control for 16 Gy (assayed at 6 hr) was 0.93% in Figure 1 and 1.94% in Figure 2. These values are much lower than for androgen ablation plus 8 Gy shown in Figure 1 (6.0%) and described previously (8).

The effect of fractionated RT on apoptosis was investigated to determine if the supra-additive apoptotic response was sustained with repeated 2-8 Gy fractions. When tumor-bearing animals were treated with repeated daily 2 Gy fractions beginning 3 days after AA and apoptosis measured 6 hr after the administration of the last fraction, there was a reduction in the level of

the supra-additive apoptotic response relative to that seen after a single 2 Gy fraction. Figure 3 illustrates that with as few as 3 daily fractions in the AA+RT treated tumors the supra-additive apoptotic response is lost. After five daily 2 Gy fractions, apoptosis in the combined treated tumors was at the level of the castrate control.

Other investigators have described that maximal recovery of the apoptotic response after single fraction radiation requires more than 24 hr (12, 13). This raised the possibility that more than 24 hr are required for recovery of the supra-additive apoptotic levels seen after one fraction. Tumor-bearing animals were castrated and 3 days later were irradiated with 2 Gy, followed 24, 48 or 96 hr later with a second fraction of the same dose. The tumors were removed and prepared for the TUNEL assay 6 hr after the second radiotherapy dose (Figure 4). Significant levels of apoptosis (>1% over controls) were not observed, even when the two fractions were spaced by 96 hr. Since the supra-additive response to AA+RT under ideal single fraction conditions was much greater with 8 Gy than 2 Gy (Figure 1), a similar experiment was performed using two fractions of 8 Gy. Figure 5 reveals that supra-additive apoptosis was not observed when two 8 Gy fractions were given, regardless of the interfraction interval. These data indicate that the first RT fraction affected the potential for supra-additive apoptosis by the second RT fraction and that recovery of the supra-additive apoptotic response requires more than 96 hr.

The Dunning R3327 model has been used in numerous studies, including some that document a radiation dose response using tumor growth delay (14-16); there is no indication that fractionating radiation would compromise tumor growth delay. Figure 6 illustrates that the tumor volume growth delay increases with increasing fractionated RT dose for the R3327-G cells used herein. The administration of ten daily 2.5 Gy fractions caused significantly more

tumor growth delay than five fractions. Previously we documented supra-additive tumor growth delay with AA plus 7 Gy, given as a single fraction, based on calculation of the enhancement factor (8). Figure 7 shows the effect of AA plus RT on tumor growth delay, as compared to the AA control and RT control. In this experiment the tumors were exposed to AA temporarily for 7 days and RT was given in four daily 2.5 Gy fractions beginning on the third post-AA day. The curves were log transformed and fitted to calculate the enhancement factor (see methods). The enhancement factor was calculated from the times for the treated tumors to reach 5 cc relative to the intact control; the intact control was taken from the composite of similar experiments done immediately before and after the one shown in Figure 7. The enhancement factor was calculated to be 1.4, which is consistent with supra-additivity. The results suggest that apoptosis was not reflective of overall cell death.

DISCUSSION

Apoptosis is believed to be an important mode of cell death in the response of prostate cancer to either AA or RT. However for prostate cancer, typical apoptotic responses to these treatments when given individually, are minimal. Whereas AA causes apoptosis in 80% of normal prostate epithelial cells (17), the apoptotic response of most malignant human (18-20) and animal (8, 9, 21) prostate tumors appears to be much lower. The apoptotic response of prostate cancers to radiation is also very low (22, 23) and under the best circumstances probably only contributes about 5% to overall cell killing. Prostate cancer is not unique in this regard, as for most tumors tested apoptosis accounts for a fraction of the loss of reproductive integrity (24).

The finding that AA and single fraction RT of 2 - 8 Gy caused supra-additive apoptosis (8) when applied in a specific sequence to prostate tumors *in vivo*, suggested that this cell death mechanism might take precedence, leading to increased overall cell killing. Confirmation of this tenet seemed apparent with the observation that enhanced apoptosis to AA plus single fraction RT was associated with supra-additive tumor growth delay. Implicit in these data is potential for considerably greater tumor eradication with fractionated radiotherapy, if supra-additive apoptosis occurred with each fraction.

Meyn and colleagues (12, 13) found that ovarian carcinoma (Oca-I) cells grown *in vitro* exhibited a significant, albeit reduced, apoptotic response when a second fraction was applied 24 hr after the first. They also observed about a 2 fold further recovery of the apoptotic response when the interfraction interval was lengthened to 5 d. A similar pattern was established by Mirkovic et al (13) using lymphoma cells grown *in vivo*.

The data described here using the Dunning R3327-G rat prostate model reveal that supra-additive apoptosis to AA plus RT is not repeated with additional fractions of γ -radiation. Nor

was a supra-additive apoptotic response evident when the interfraction interval between two fractions of 2 or 8 Gy was extended to 96 hr (Figures 4 & 5). No recovery of the supra-additive apoptotic response was noted within the limits of the study. Thus, the reduction in apoptosis under these conditions appears not to be consistent with changes in the distribution of cells in the cell cycle caused by split-dose irradiation (25, 26). We chose not go beyond 96 hr because such extended times would be impractical in designing a clinical regimen that optimized the apoptotic response. The level of cell killing evidenced by apoptosis after multiple fractions was not concordant with the tumor growth delay results. Figure 7, and the resulting calculations of the enhancement factor, demonstrate that the combination of AA with multiple RT fractions causes a greater delay in tumor growth than the addition of the individual effects of AA and RT. Supra-additive tumor growth inhibition with AA + RT is probably related to an increase in overall cell death; however, an alternative mechanism is that tumor proliferation was suppressed. The former explanation, that AA + RT causes increased overall cell killing via necrosis, is supported by the reduction in TCD₅₀ observed under these conditions in this cell line (5).

The single fraction dose-response experiment shown in Figure 1 provides additional evidence that apoptosis levels after AA + RT appear to be contrary to the expected dose-response achieved with measures of overall cell killing, such as tumor growth delay (14). Irradiation with AA plus 16 Gy resulted in a lower apoptotic index than AA plus 8 Gy. In contrast, tumors treated with radiation alone had consistently higher levels of apoptosis when the dose was increased from 8 Gy to 16 Gy, which is the dose-response pattern that others have reported (12, 13).

Apoptosis is clearly a secondary mechanism of cell killing from irradiation (24, 27). Our data show that apoptosis is suppressed with the administration of multiple fractions in the setting

of androgen deprivation. Likewise, the apoptotic index was lower to AA plus 16 Gy given in a single dose, as compared to AA plus 8 Gy. The administration of AA plus RT alters the relationship of apoptosis to overall cell killing, suggesting that AA affects the expression of key proteins in the apoptotic pathway or that the impact of known proteins is diminished by changes in the expression of less characterized downstream factors. Along these lines, Kyprianou et al (28) have observed that bcl-2 overexpression delays radiation induced apoptosis without affecting clonogenic survival. From these results and those described here with AA plus fractionated RT, it would appear that under certain conditions the level of apoptosis is not reflective of overall cell death.

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FIGURE LEGENDS

- Figure 1. Supra-additive apoptotic dose-response for R3327-G tumors grown in castrated rats for 3 days, irradiated, and then removed for TUNEL staining 6 hr later (Solid circles). The intact irradiated RT alone controls are shown (open squares).
- Figure 2. Effect of varying the time after AA plus 16 Gy irradiation on measurable apoptosis. Tumor-bearing rats were castrated, irradiated 3 days later with 16 Gy, and examined for apoptosis at different times thereafter (Solid circles).
- Figure 3. R3327-G tumor apoptotic response to 1, 3, or 5 daily 2 Gy fractions. Tumor-bearing rats were castrated, irradiated with the first fraction 3 d later, and removed 6 hr after the last fraction (Solid circles). The following controls are shown: RT alone = open squares; Intact unirradiated control = open diamonds; AA Alone = Solid triangles.
- Figure 4. Effect of varying the interval between two fractions of 2 Gy on the apoptotic response of R3327-G tumors grown for 3 days in castrate rats (Solid circles). Apoptosis was measured 6 hr after the second fraction. The intact controls are also shown (Open circles).
- Figure 5. Effect of varying the interval between two fractions of 8 Gy on the apoptotic response of R3327-G tumors grown for 3 days in castrate rats (Solid circles).

Apoptosis was measured 6 hr after the second fraction. The intact controls are also shown (Open circles).

Figure 6. R3327-G tumor volume growth in intact rats after no treatment (solid circles), 2.5 Gy daily for 5 fractions (solid squares), and 2.5 cGy daily for 10 fractions (solid triangles). The experiment was terminated at 65 days and at this time there were no deaths in the 2.5 cGy x 10 group.

Figure 7. R3327-G tumor volume growth in rats castrated and 3 days later irradiated with 2.5 Gy daily for four fractions (AA + 2.5 Gy x 4, solid triangles), as compared to AA alone (solid circles) and RT alone (2.5 Gy x 4, open squares). The castrated animals underwent androgen restoration with a testosterone implant one week after castration.

Figure 1, Pollack et al

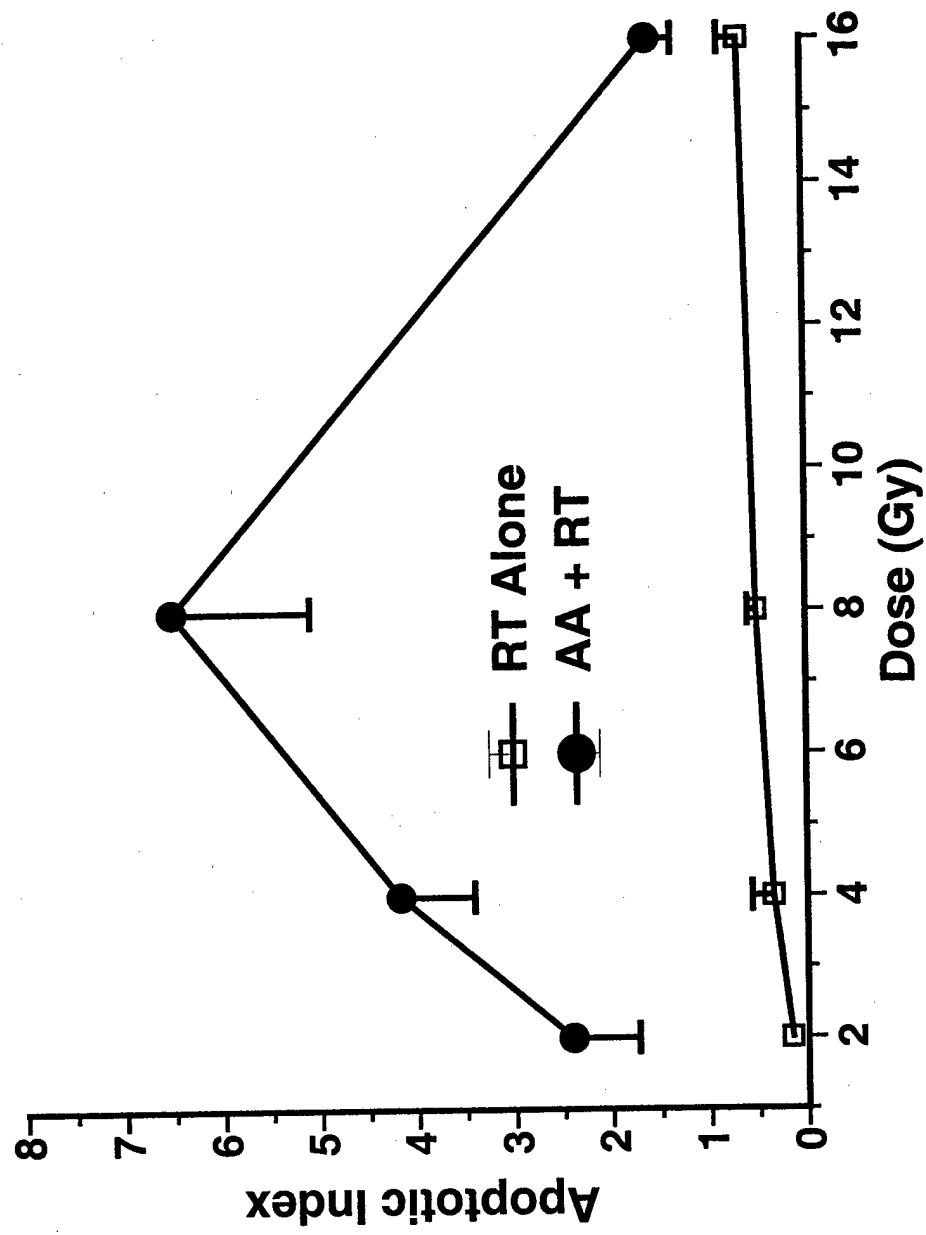


Figure 2, Pollack et al

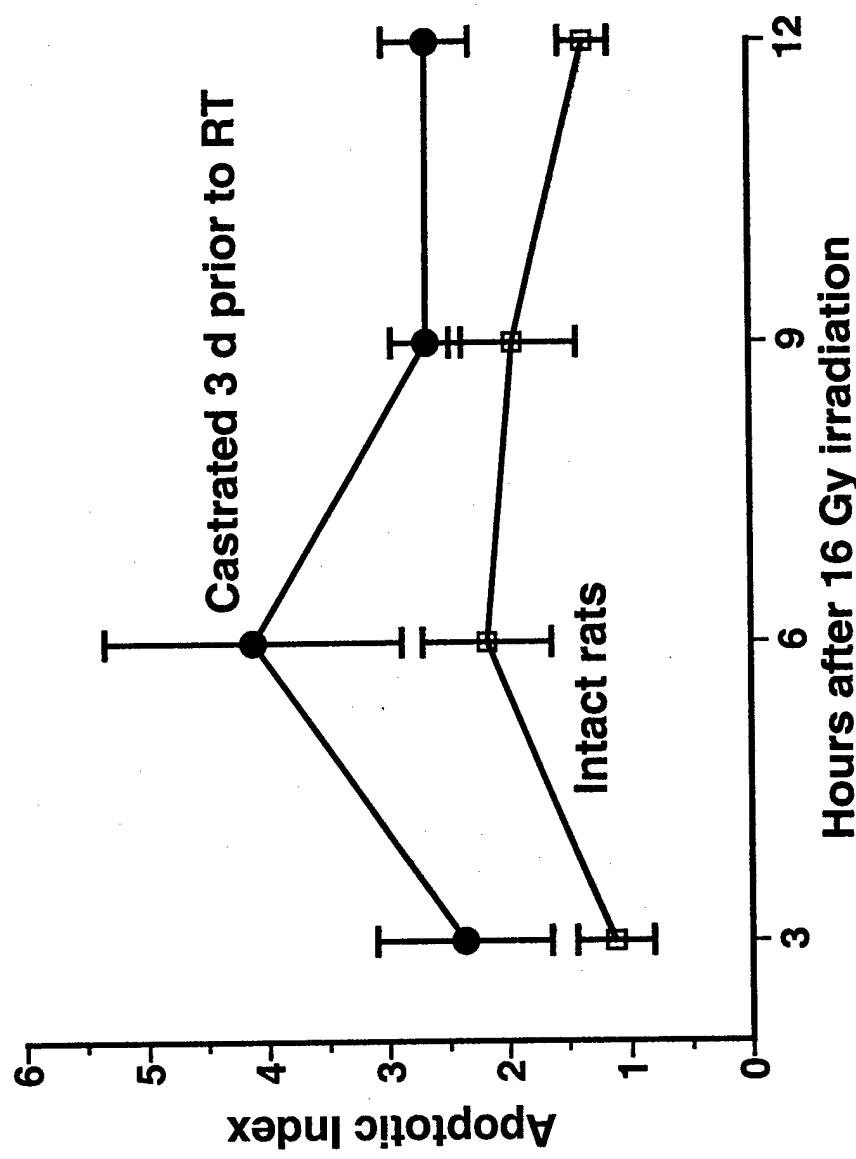


Figure 3, Pollack et al

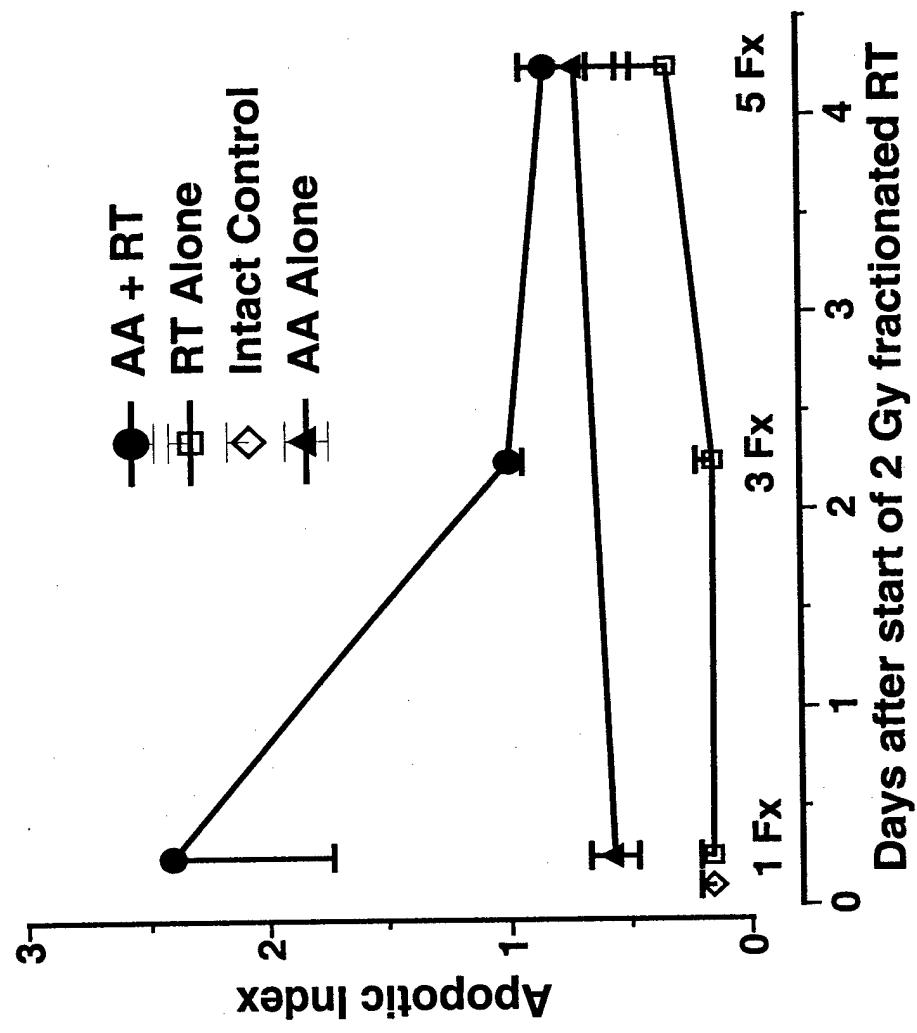


Figure 4, Pollack et al

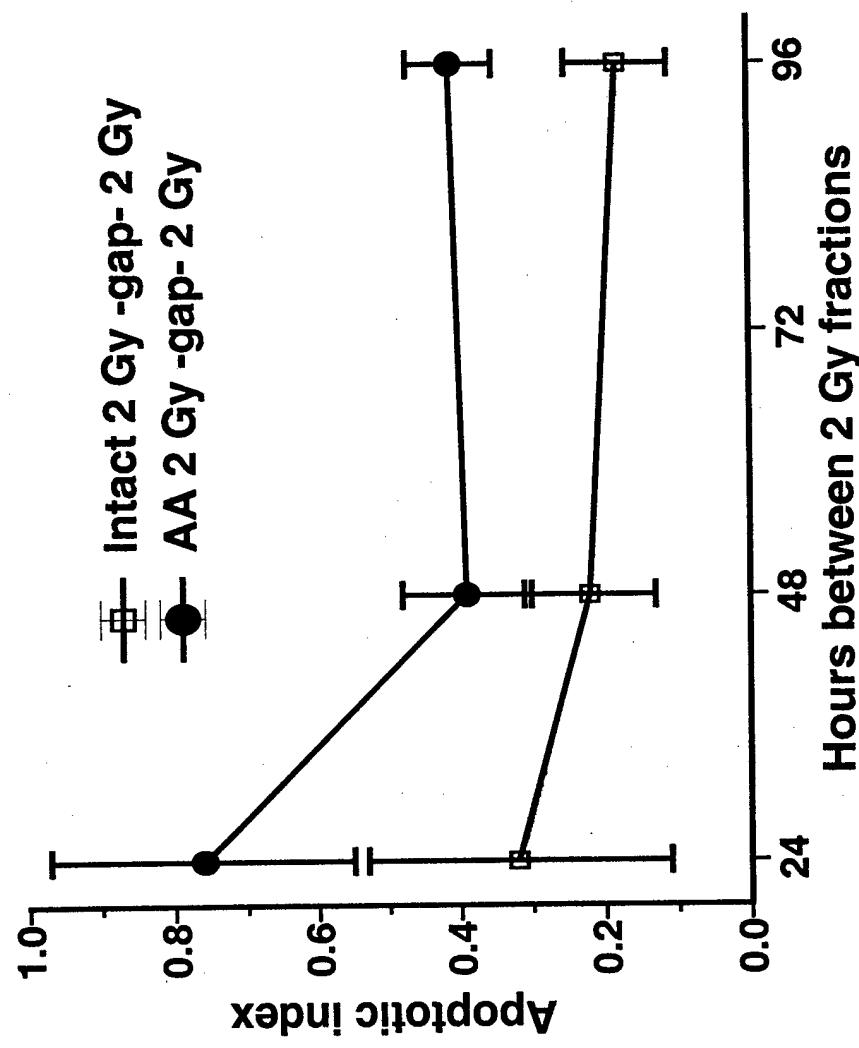


Figure 5, Pollack et al

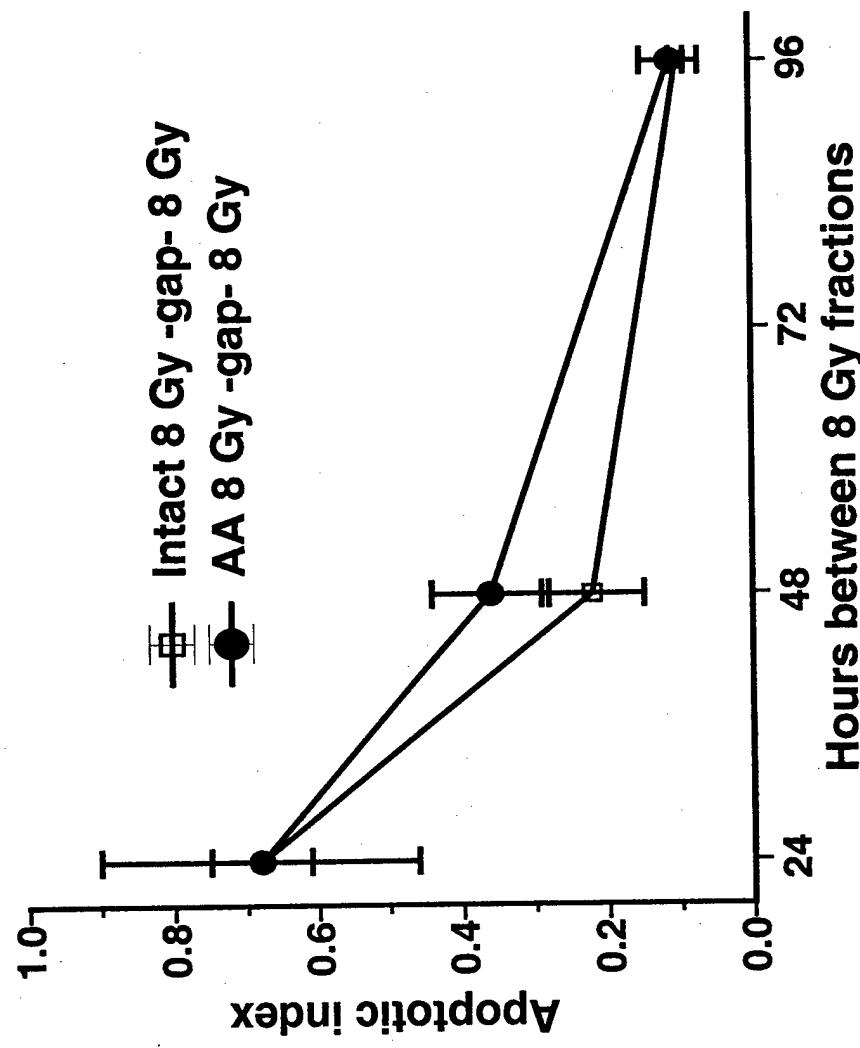


Figure 6, Pollack et al

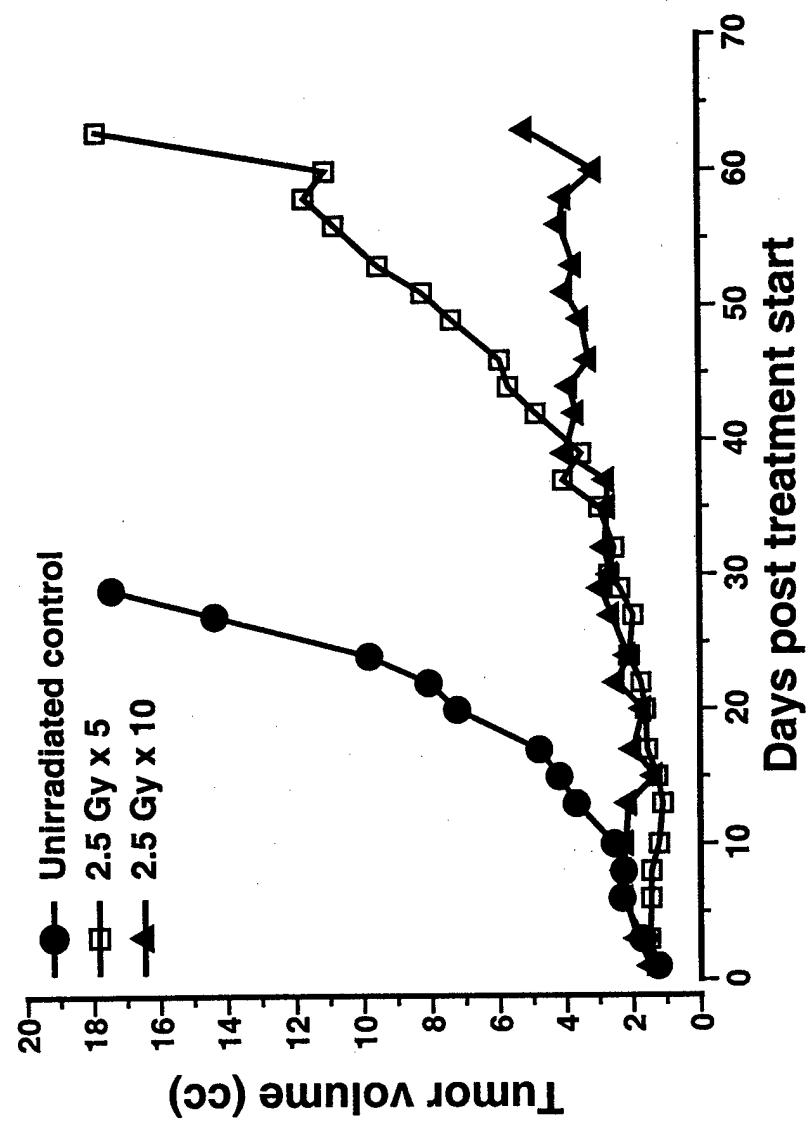
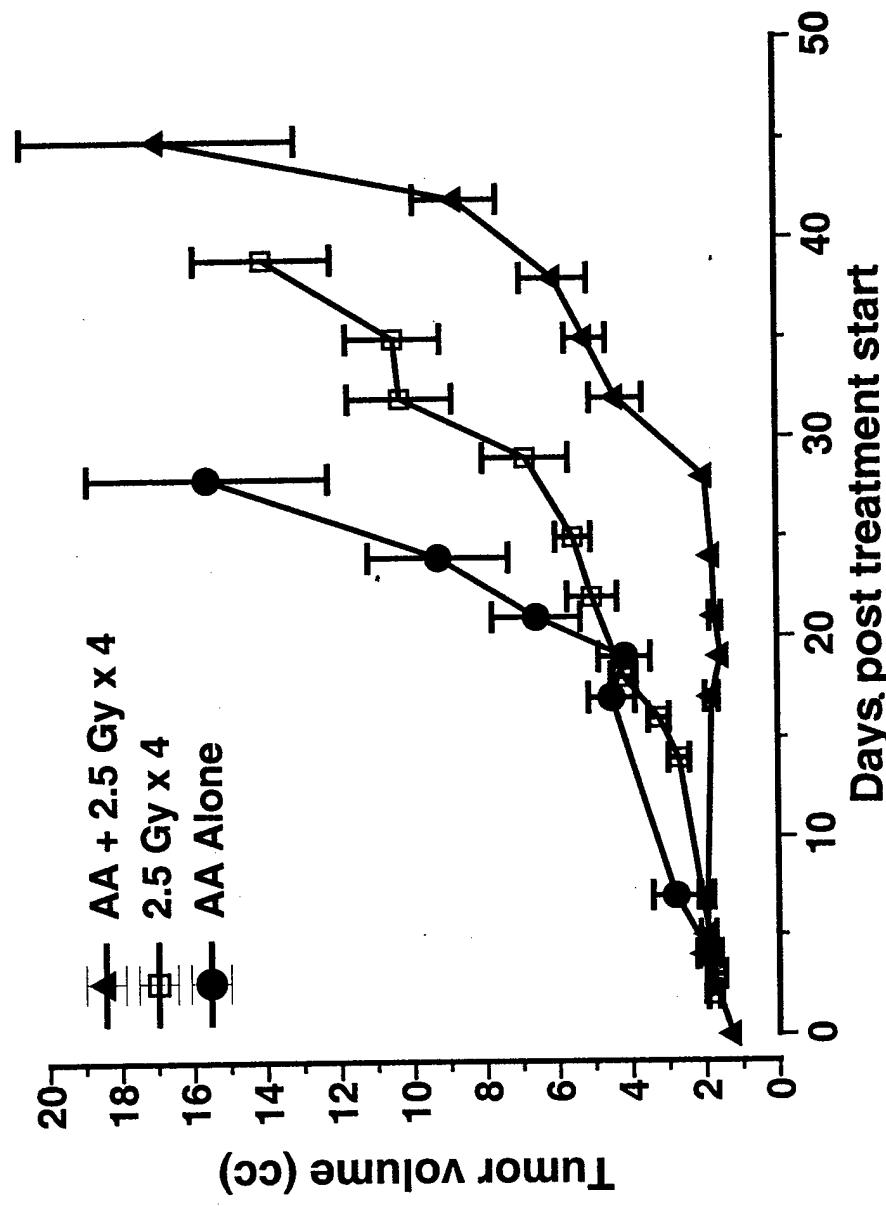


Figure 7, Pollack et al



Pollack, Alan...1

**RELATIONSHIP OF KI-67 LABELING INDEX TO DNA-PLOIDY, S PHASE FRACTION,
AND OUTCOME IN PROSTATE CANCER TREATED WITH RADIOTHERAPY**

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98-214

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ABSTRACT

Purpose: To evaluate the relationship of Ki-67 labeling index (Ki67-LI) to deoxyribonucleic acid (DNA) ploidy, S phase fraction (SPF), other clinical prognostic factors, and clinical outcome for patients with prostate cancer treated by external beam radiotherapy.

Materials and Methods: Tissue was retrieved from 42 patients who underwent transurethral resection of the prostate before treatment with external beam radiotherapy between 1987 and 1993. DNA histogram profiles were classified as diploid (diploid + near-diploid) and nondiploid (tetraploid + aneuploid). Immunohistochemical staining of Ki-67 by the MIB-1 monoclonal antibody was used to calculate Ki67-LI. Median patient follow-up was 62 months. Treatment failure was defined as two consecutive rises in serum prostate specific antigen (PSA) or clinical evidence of disease recurrence.

Results: The mean and median Ki67-LIs were 3.1 and 2.4, respectively (range 0 - 12.4). Mean Ki67-LI values were significantly associated with higher stage, Gleason score, and pretreatment PSA. Nondiploid tumors had significantly higher Ki67-LIs, as did patients who failed radiotherapy over the follow-up period. SPF was not significantly correlated with Ki67-LI. As a categorical variable, the most significant relationships were seen when Ki67-LI was subdivided into thirds around the median (Ki67-LI \leq 1.5, Ki67-LI >1.5-3.5, and Ki67-LI >3.5%). This trichotomous variable correlated significantly with pretreatment PSA ($p = 0.0008$), tumor stage ($p = 0.016$) Gleason score ($p = 0.024$), and treatment failure ($p = 0.0015$), but not with DNA ploidy ($p = 0.15$). In actuarial univariate analyses, Ki67-LI appeared to be a more significant predictor of patient outcome ($p = 0.003$) than DNA ploidy ($p = 0.035$).

Conclusions: The Ki67-LI correlated with known prognostic factors such as pretreatment PSA, tumor stage, and Gleason score, and was also weakly related to DNA ploidy. In comparison to DNA ploidy, Ki67 LI seems to be a better correlate of treatment outcome.

Running Title: Prostate cancer Ki-67 labeling index and DNA ploidy

Key words: DNA ploidy, Ki-67, MIB-1, Prostate cancer, Prostate specific antigen, Radiotherapy.

INTRODUCTION

The deoxyribonucleic acid (DNA) content of prostate tumors has repeatedly been shown to be predictive of disease outcome (1-15). In our experience (16-18), DNA ploidy is an independent correlate of biochemical and/or clinical failure after radiotherapy for clinically localized prostate cancer. The potential of DNA ploidy for enhancing the prognostic classification of prostate cancer is evident from these studies; however, the application of such measurements clinically has been limited. Flow cytometry and image analysis, the two most common methods for quantifying DNA content, are technically demanding methods. The classification of the histograms into diploid, tetraploid, and aneuploid is highly variable between investigators and not entirely objective. Some improvement in the resolution of overlapping cell populations is obtained by analyzing DNA in combination with other parameters, such as nuclear protein (17), but, this adds complexity to an assay already difficult to standardize.

Immunohistochemical staining of the proliferation marker, Ki-67, has been shown to reasonably approximate growth fraction in prostate cancers and other malignancies (19-22). In contrast, DNA content histograms are strictly a freeze-frame of the proportion of cells distributed about the cell cycle phases. Although such histograms provide an approximation of the fraction of cells in S phase (SPF), the Ki-67 labelling index (Ki67-LI) is a more functional estimate of proliferation. The relationships between Ki67-LI and the DNA content parameters of DNA ploidy and SPF are poorly documented for prostate cancer (23-25). In addition, a number of reports have indicated that Ki67-LI is significantly related to prostate cancer patient outcome after radical prostatectomy or androgen ablation therapy (26-32). Preliminary results in radiotherapy treated patients are also encouraging (33). The purpose of this report is to explore the correlation of the

DNA content parameters and Ki67-LI, and to determine the relative predictive value of these factors for the outcome of patients treated with radiotherapy.

MATERIALS AND METHODS

Patient characteristics

Sections from transurethral resection of the prostate (TURP) specimens were used for this study because the tissue requirements for MIB-1 immunohistochemical staining and DNA ploidy by flow cytometry were beyond that of most needle biopsy specimens. There were 151 patients with prostate cancer diagnosed from TURP that were referred to MDACC between 1987 and 1993. Of these patients, paraffin-embedded prostatic sections were obtained from 42 patients. All patients were treated with definitive radiotherapy only; no patient received neoadjuvant or adjuvant androgen ablation, underwent radical prostate surgery, or underwent surgical lymph node dissection. The workup of patients treated with radiotherapy at MDACC has been described previously (34).

Pretreatment serum PSA levels were determined in all patients. The median and mean pretreatment PSAs were 4.1 and 8.8 ng/ml, respectively (range 0.3-92 ng/ml). The median and mean age was 68 years (range 56 - 79 years). The median follow-up was 62 months (range 19 - 121 months). The clinical stages for the study population were: Stage T1 in 27 patients (64%); Stage T2 in 8 (19%); Stage T3 in 6 (14%); and Stage T4 in 1 (2%). The Gleason scores were: Gleason score 5 in 6 patients (14%); Gleason score 6 in 15 (36%); Gleason score 7 in 13 (31%); Gleason score 8 in 3 (7%); Gleason score 9 in 4 (10%); and Gleason score 10 in 1 (2%).

The median external beam radiotherapy dose was 64 Gy, with a mean of 65 Gy and a range of (60 - 78 Gy). Radiotherapy was delivered via a four field box with 18 MV photons using a shrinking field technique in all but one patient, who received a conformal 6 field boost after 46 Gy to a total dose of 78 Gy (34). The dose was specified to the isocenter at 2 Gy per day. After the completion of radiotherapy, patients were followed at 3-6 month intervals with history, clinical examination, and repeat serum PSA for 2 years and then every 6-12 months thereafter.

Biochemical failure (a rising PSA profile) was defined as two or more consecutive rising PSA values following the post-radiotherapy PSA nadir. The actuarial curves for the incidence of PSA rise were calculated from the average time between the nadir PSA value and the first elevated PSA value.

Immunohistochemistry technique

The monoclonal antibody, MIB-1, was used as a proliferation marker (Immunotech, SA MAC Inc., Germany). The slide-mounted paraffin-embedded prostatic tissue sections were deparaffinized in xylene before being rehydrated sequentially in ethanol (100%, 90%, 70%) and placed into a 1% phostate buffered solution (PBS, pH 7.4). The sections were heated in a conventional 600 W. microwave oven at maximum power for 3 x 5 minutes. The sections were then allowed to cool to room temperature for 40 minutes before using 2% normal horse serum to block non-specific protein binding. The sections were incubated with MIB-1 antibody (1:50) overnight at 4°C in a humidified chamber. Detection of the bound MIB-1 antibody involved applying the VECTASTAIN Elite ABC reagents (Vector Laboratories Inc, Burlingame, CA, USA) using Avidin DH: biotinylated horseradish peroxidase H complex with 3,3'-diaminobenzidine (Polysciences Inc., Warington, PA) and Mayer's hematoxylin (Fisher Scientific, Fair Lawn, New Jersey). Appropriate positive controls (HeLa cells) were included in each immunohistochemical run to verify the specificity of MIB-1 and negative controls were produced by substituting the primary antibody with PBS in duplicate sections.

Tissue specimens and MIB-1 grading

All original TURP diagnostic material and additional sections were reviewed by the study pathologist (P.T.) and graded according to the Gleason system. Sections representative of the tumor

with the highest grade were selected for immunohistochemistry. Within the selected tumor regions, random fields measuring at least 500 tumor cells were assessed using an eyepiece graticule at 400 \times magnification. Positive MIB-1 cells were counted by scoring any appropriate tumor nuclei staining regardless of its intensity. The labeling index of MIB-1 was expressed as a percentage of immunoreactive cells to the counted tumor cells. Scoring of these sections were performed by two of the investigators (V.K. and D.C.) without any prior knowledge of the patient data or treatment related outcomes. The averages of these independent counts were used for the analyses.

Flow cytometric sample preparation and analysis

Paraffin embedded tissue was prepared for flow cytometry using a method previously described in detail (16,17). Briefly, 1-2 sections of 50 μm were deparaffinized with xylene, rehydrated with graded alcohols, digested in pepsin, and the nuclei isolated using nuclear isolation buffer (0.5% Nonidet P40; 0.05 M Trizma base: Trizma-HCl pH 7.4; 0.05 M NaCl; 1 mM EDTA.). The protein in the extracted nuclei was stained with fluorescein isothiocyanate (FITC) and the DNA with propidium iodide (PI). Analyses of the samples were performed using a EPICS 752 flow cytometer (Coulter Electronics, Hialeah, FL) with an argon-ion laser set at 488 nm. Appropriate filters were used to resolve the green FITC signals from the red PI signals. The resultant DNA/nuclear protein histogram profiles were classified as diploid ($n = 18$), near-diploid ($n = 9$), tetraploid ($n = 5$), or aneuploid ($n = 6$), based on previously defined criteria (17). Because the number of patients was small and we have shown (17) that the greatest differences for similar patients is between diploid/ near-diploid (termed "diploid" here) versus tetraploid/aneuploid (termed "nondiploid" here), these groups were used for the analyses described below.

As described previously, S-phase fraction (SPF) was derived from the single parameter DNA histograms using MODFIT-LT software (18). The model corrected for single-cut debris, approximated S-phase using rectangles, and estimated G1 and G2M using gaussians. A single composite SPF for the normal and tumor populations was determined when the DI was <1.3. In aneuploid or tetraploid cases, the SPFs of both populations was estimated; but, only the aneuploid/tetraploid SPF was used in subsequent comparisons.

Statistics

The chi-square test was used to assess the significance of differences between proportions (35). Non-parametric comparisons between independent groups were performed using the Mann-Whitney test. Actuarial curves were calculated using the Berkson-Gage method with tests of statistical significance based on the log-ranked statistic (36).

RESULTS

Table 1 shows the relationship of the mean Ki67-LI values to the potential prognostic factors of the study cohort. The groupings of PSA, Gleason grade, tumor stage, and DNA ploidy were based on previous studies (16-18, 34). The mean Ki67-LI was 3.1%, with a median of 2.4% and a range of 0 - 12%. Significantly higher mean Ki67-LI values were seen with Stage T3/T4 disease, Gleason score ≥ 7 , pretreatment PSA >10 , or nondiploidy. No association was seen between SPF Gleason score ≥ 7 , pretreatment PSA >10 , or nondiploidy. No association was seen between SPF Gleason score ≥ 7 , pretreatment PSA >10 , or nondiploidy. Treatment failures correlated with higher Ki67-LIs. (stratified by the median value) and Ki67-LI. Treatment failures correlated with higher Ki67-LIs.

As a categorical dichotomous variable, stratified around the median value, Ki67-LI was a correlate of palpable stage, Gleason score, and pretreatment PSA (Table 2). A weaker, borderline significant, association was found with DNA-ploidy. With the exception of DNA-ploidy, these correlations were more significant when the patients were divided into thirds based on Ki67-LI as a trichotomous variable (Table 3). High Ki67-LIs above 3.5% were seen in significantly more patients with T3/T4 disease, Gleason scores ≥ 7 , and pretreatment PSAs > 10 . Of the patients that failed biochemically, 62% had a Ki67-LI $>3.5\%$.

The relationship of Ki67-LI with actuarial biochemical failure is shown in Figure 1. Ki67-LI predicted for failure when used as either a dichotomous or trichotomous variable. The most significant correlation was seen with the latter (Table 4), in which no failures were evident at 4 years if the Ki67-LI was $\leq 1.5\%$ and 33% failed if the Ki67-LI was $>3.5\%$. Only pretreatment PSA was a more significant determinant of failure. When Cox proportional hazards regression was performed, the only independent correlate of failure was pretreatment PSA.

DISCUSSION

The development of MIB-1 for the staining of Ki-67 antigen in formalin fixed tissues (37) has promoted the exploration of this marker of cell proliferation as a potential prognostic factor for patients with prostate cancer. Studies examining the relationship of Ki67-LI to other prognostic factors and patient outcome have, in general, indicated that this parameter may be useful in addition to Gleason score and stage. The majority of investigators have found that Ki67-LI correlates with tumor grade and stage (21,26,27,32,39-41). In some reports these relationships were of borderline significance (42). Rarely, no correlations with other prognostic factors were found (24). For the cohort of TURP-diagnosed prostate cancer patients we investigated, highly significant associations between Ki67-LI and Gleason score, palpable stage, and pretreatment PSA were identified. Patients with high risk features of Gleason score ≥ 7 , stage T3/T4 disease, or pretreatment PSA > 10 ng/ml had higher mean Ki-67-LIs and were comprised of a greater percentage with Ki67-LIs $> 3.5\%$.

The Ki67-LI is a static immunohistochemical estimate of tumor growth fraction (19-22). Since a significant proportion of proliferating cells are in S phase, one would expect that the SPF obtained by flow cytometry would correlate with Ki-67-LI (41, 43). The data presented did not reveal any relationship between Ki67-LI and SPF. The discrepancy between Ki-67-LI and SPF is probably due in part to the difficulty in separating the normal epithelial and stromal cells from the tumor cells in the resultant flow cytometric DNA histograms. Although some investigators have found SPF to have potential as a correlate of patient outcome (15), we have never found SPF to be useful (16-18).

In terms of DNA ploidy, a borderline significant relationship with Ki67-LI was seen (Tables 1-3). The 11 nondiploid tumors had a higher mean Ki67-LI. Coetzee et al (24), Cher et al (23), and Uzoaru et al (25) also reported associations between Ki67-LIs and DNA ploidy. These results indicate that the parameters of Ki67-LI and DNA ploidy are significantly, albeit weakly, related.

Several studies have examined the prognostic importance of Ki67-LI in patients with prostate cancer. In nearly every report, Ki-67-LI has been predictive of patient outcome in actuarial univariate analyses. While the majority have confirmed the independence of Ki67-LI as a correlate of patient outcome in multivariate analysis (26, 27, 29, 32, 33, 42), others have not (24). The number of patients in our study ($n = 42$) was inadequate to accurately assess the independence of Ki67-LI as a predictor of freedom from failure. Pretreatment PSA was the only correlate by Cox proportional regression in this series. Prior studies with larger numbers of patients have established that Gleason score, clinical stage, and DNA ploidy are also independent correlates (16, 17, 34).

CONCLUSION

The Ki67-LI is significantly related to other prognostic factors, such as pretreatment PSA, Gleason score, and stage, and is a strong predictor of patient outcome. The data suggest that Ki67-LI is a stronger correlate of prostate cancer patient outcome following radiotherapy than DNA ploidy or SPF. A Ki67-LI of $>3.5\%$ was associated with a particularly poor prognosis. Prospective evaluation of pretreatment prostate tumor biopsy Ki67-LI will help to clarify the role of this potentially useful cell kinetic marker.

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Table 1. Percent Ki-67 staining by various potential prognostic factors.

<u>Grouping</u>		N	<u>Mean ± SE</u>	<u>p*</u>
All Patients		42	3.1 ± 0.4	-----
Stage	T1/T2	35	2.4 ± 0.3	
	T3/T4	7	6.7 ± 1.4	0.003
Gleason Score	2-6	21	2.0 ± 0.3	
	7-10	21	4.2 ± 0.7	0.017
Pretreatment PSA	≤ 10	33	2.2 ± 0.3	
	> 10	9	6.4 ± 1.0	0.0003
DNA Ploidy	Diploid	27	2.5 ± 0.4	
	Nondiploid	11	4.7 ± 1.1	0.04
Percent S Phase	≤ 2.5	14	2.9 ± 0.8	
	> 2.5	15	3.2 ± 0.7	0.53
Treatment Failure	No	29	2.3 ± 0.4	
	Yes	13	4.9 ± 0.7	0.0009

*Mann - Whitney test; SE = standard error.

Table 2. Distribution of patients by Ki-67 staining as a dichotomous variable.

		% Ki-67		<u>p*</u>
		<u>≤ 2.4</u>	<u>> 2.4</u>	
<u>Grouping</u>				
Stage	T1/T2	54 (19)	46 (16)	0.05
	T3/T4	14 (1)	86 (6)	
Gleason Score	2-6	67 (14)	33 (7)	0.01
	7-10	29 (6)	71 (15)	
Pretreatment PSA	≤ 10	61 (20)	39 (13)	0.001
	> 10	0 (0)	100 (9)	
DNA-Ploidy	Diploid	59 (16)	41 (11)	0.07
	Nondiploid	27 (3)	73 (8)	
Percent S Phase	≤ 2.5	57 (8)	43 (6)	0.57
	> 2.5	47 (7)	53 (8)	
Treatment Failure	No	62 (18)	38 (11)	0.005
	Yes	15 (2)	85 (11)	

*Chi-square

Table 3. Distribution of patients stratified by Ki-67 staining as a trichotomous variable.

<u>Grouping</u>		<u>Percent (n) Patients By Ki-67*</u>			<u>p**</u>
		<u>≤1.5</u>	<u>>1.5 – 3.5</u>	<u>>3.5</u>	
All					
Stage	T1/T2	37 (13)	43 (15)	20 (7)	
	T3/T4	0 (0)	29 (2)	71 (5)	0.016
Gleason Score	2-6	38 (8)	52 (11)	10 (2)	
	7-10	24 (5)	29 (6)	48 (10)	0.024
Pretreatment PSA	≤ 10	40 (13)	46 (15)	15 (5)	
	> 10	0 (0)	22 (2)	78 (7)	0.0008
DNA-Ploidy	Diploid	41 (11)	37 (10)	22 (6)	
	Nondiploid	9 (1)	46 (5)	46 (5)	0.15
Percent S Phase	≤2.5	36 (5)	36 (5)	29 (4)	
	>2.5	27 (4)	40 (6)	33 (5)	0.87
Treatment Failure	No	45 (13)	41 (12)	14 (4)	
	Yes	0 (0)	39 (5)	62 (8)	0.0015

* Patients were stratified by %Ki-67 staining of ≤1.5%, >1.5 – 3.5%, and >3.5%.

**Trended Chi-square

Table 4. Correlates of freedom from biochemical failure.

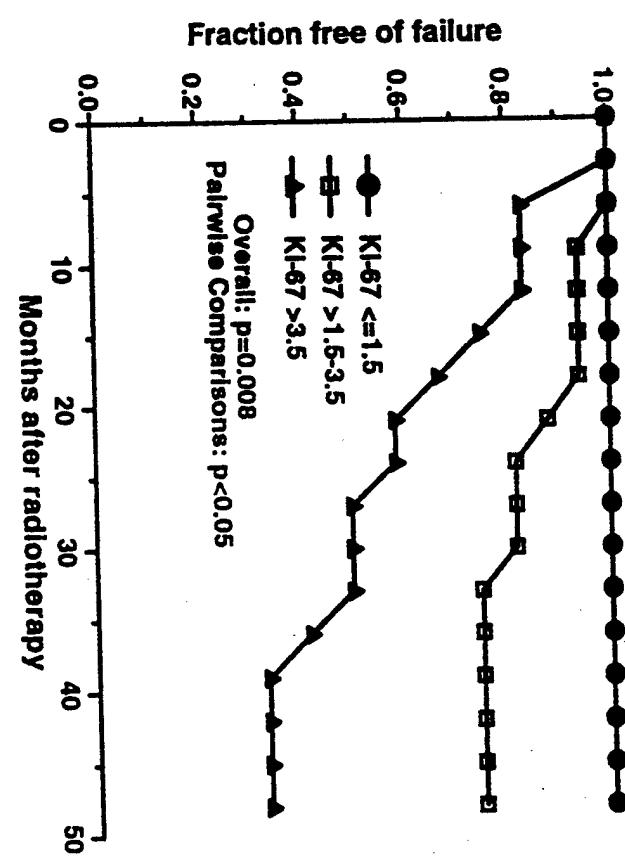
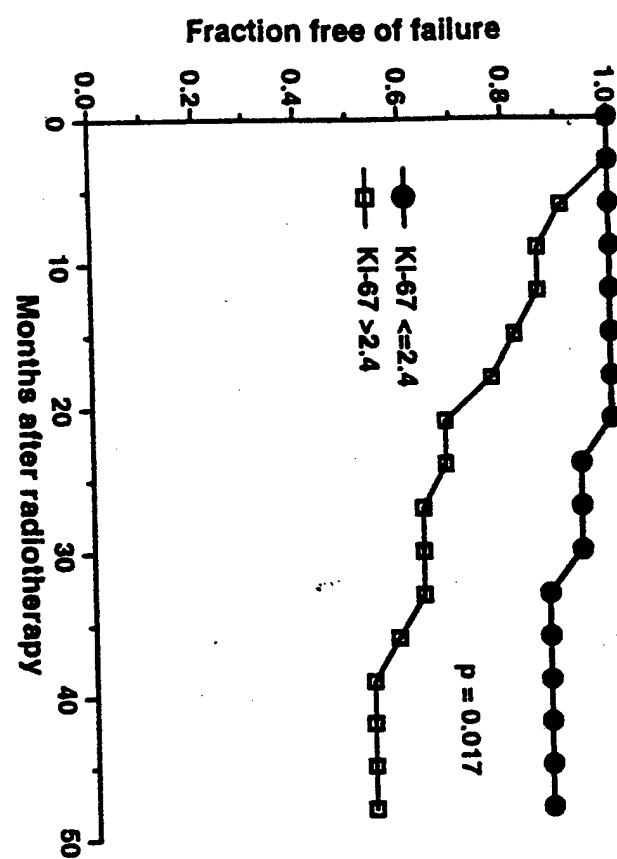
<u>Factor</u>		<u>4 year % Free of failure</u>	<u>p*</u>
Stage	T1/T2	78	0.005
	T3/T4	< 29†	
Gleason Score	2-6	90	0.002
	7-10	47	
Pretreatment PSA	≤ 10	86	< 0.0001
	> 10	< 11†	
DNA-Ploidy	Diploid/Near-diploid	75	0.035
	Aneuploid/Tetraploid	45	
Percent S-Phase	≤ 2.5	78	0.46
	> 2.5	62	
Ki67-LI	≤ 2.4	88	0.017
	> 2.4	54	
Ki67-LI	≤ 1.5	100	0.003
	> 1.5-3.5	75	
	> 3.5	33	

*Log-rank test

†The less than sign indicates that the number of patients at risk at 4 years was small and the percentage shown less accurate.

Figure Legends

Figure 1. Relationship of Ki67-LI as a dichotomous (left panel) and a trichotomous (right panel) variable to actuarial freedom from failure. The number of patients at risk at 24 and 48 months were 17 and 7 for Ki67-LI $\leq 2.4\%$, 15 and 11 for Ki67-LI $> 2.4\%$, 11 and 5 for Ki67-LI $\leq 1.5\%$, 14 and 9 for Ki67-LI $> 1.5 - 3.5\%$, and 7 and 4 for Ki67-LI $> 3.5\%$.



**ADENOVIRAL – MEDIATED p53 TRANSGENE EXPRESSION SENSITIZES BOTH
WILD-TYPE AND MUTANT p53 PROSTATE CANCER CELLS
IN VITRO TO IRRADIATION**

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Running Title: p53 Mediated Radiosensitization

ABSTRACT

Purpose/Objective: The effect of adenoviral mediated p53 transgene expression on the radiation response of two human prostate cancer cell lines, the p53^{wildtype} LNCaP and p53^{null} lines, was examined. The objective was to determine if this vector sensitizes cells to radiation independently of their p53 status.

Materials & Methods: A recombinant adenovirus-5 vector containing a CMV promoter and wild-type p53-cDNA (Ad5-p53) was used to facilitate p53 transgene expression. A multiplicity of infection of 40-70 viral particles per cell was used, based on Ad5-lacz infection and staining for the β-galactosidase reporter gene product. Clonogenic assays were performed to evaluate the degree of sensitization to radiation of viral-transfected cells compared with irradiated non-transfected controls. The effect of these conditions on cell death via apoptosis was determined using the TUNEL assay.

Results: The delivery of Ad5-p53 reduced control plating efficiency from 42% to 1.5% in the LNCaP cell line and from 88.6% to 0.55% in the PC3 cell line. After correcting for the effect of Ad5-p53 on plating efficiency, the surviving fraction after 2 Gy (SF2) of gamma-irradiation was reduced over 2 fold, from 0.197 to 0.063 with transgene p53 expression in the LNCaP cell line. Survival after 4 Gy was reduced 4.5 fold, from 0.011 to 0.002, after Ad5-p53 treatment. In the PC3 cell line, Ad5-p53 reduced the SF2Gy over 0.6 fold from 0.603% to 0.369%, and the SF4 over 48 fold from 0.246% to 0.005. In both the LNCaP and PC3 cell lines, the combination of Ad5-p53 (25 MOI) plus radiation (2 Gy) resulted in supra-additive apoptosis (34.1% for LNCaP and 18.2% for PC3), above that seen from control vector Ad5-pA plus RT (0.5% for LNCaP and

9.4% for PC3), Ad5-p53 alone (0.6% for LNCaP and 1.4% for PC3), RT alone (0% for LNCaP and 0.03% for PC3), Ad5-pA alone (0.03% for LNCaP and 0% for PC3)..

Conclusion: The clonogenic survival and apoptosis data show that p53 transgene expression sensitizes human prostate adenocarcinoma cells *in vitro* to irradiation. This effect was observed in both the p53^{wildtype} LNCaP and p53^{null} PC3 lines.

Key Words: gene therapy, p53, radiotherapy, prostate cancer, apoptosis

INTRODUCTION

The process by which irradiated cells die has been shown to involve two principal mechanisms: mitotic and apoptotic cell death. The importance of apoptosis as a mechanism of radiation-induced cell death has been found to vary greatly according to cell type, being most prevalent in lymphomas and essentially absent in sarcomas (1). Apoptosis is also present as a cause of cell death in irradiated normal tissues. In prostate cancer, early apoptosis at 3 – 24 hr after radiation does not appear to be the dominant mechanism of cell death (2,3). Although current data suggest that apoptosis is a secondary mechanism of cell death from radiation, alterations in the expression of proteins that regulate this pathway have been associated with resistance to treatment and the development of hormone refractory disease (4,5). The hypothesis that formed the basis for the studies described here was that prostate cancer cell death could be enhanced overall by manipulating the intracellular molecular milieu such that apoptosis was enhanced.

Apoptosis has been found to be variably dependent on the presence of the tumor suppresser gene p53 (6-10). The response of most cells to ionizing radiation is characterized by a rise in the level of p53 within hours of treatment. This increase in p53 precedes G1 arrest and apoptosis, and p53 has been found to have a central role in these responses. Transfection of p53 mutant cells with wild-type p53 plasmids has induced both G1 arrest and apoptosis in the absence of other stressors such as chemotherapy and radiation (11,12). Enhancement of p53 expression through gene therapy was shown to induce apoptosis in several cell lines, including prostatic carcinomas. These findings have naturally led to intensive investigation as to whether replacement of p53^{wildtype} status, and the attendant control of the cell cycle, might restore

apoptosis and enhance radiation response via this mechanism of cell death. In this report we investigate whether p53 transgene expression is effective at sensitizing p53^{wildtype} and p53^{null} human prostate cell lines, and whether apoptosis is a major cell death mechanism under these conditions.

METHODS AND MATERIALS

Cell Culture. The p53^{wildtype} LNCaP and p53^{null} PC3 prostate cancer cell lines were chosen to study the effects of transgene p53 expression on radiation response. These cell lines were obtained from the American Type Culture Collection. All cells were maintained in DMEM/F12 supplemented with 10% Fetal Bovine Serum, 1% of 200 mM L-glutamine, 1% of 10,000 IU/ml Pen-Strep solution, and incubated in a 5% CO₂ incubator at 37 degrees Celsius.

Adenoviral Vector. The Ad5-p53 adenoviral vector used in this experiment has been described previously (13). The p53 expression cassette consists of a genome 35.4 kb in size. The replication defective vector includes a human cytomegalovirus (CMV) promoter, human wild-type p53 cDNA, simian virus 40 early polyadenylation signal, two cDNA- specific primers, and two viral genome-specific primers. This replaces the E1 region of the Ad5 genome. Prior to transfection, purified virus was aliquoted so that all virus had been subjected to the same number of freeze-thaw cycles. Infection of cell lines was accomplished by dilution of viral stock to the multiplicity of infection (MOI) value of 25 – 70 viral particles per cell, based on infection with Ad5/CMV/lacZ and staining for the B-galactosidase reporter gene product (14). This Ad5-βgal

vector was utilized in some experiments as a control vector. We also used a polyadenylation sequence only vector (Ad5-pA) as a control in some experiments.

Gene Transfection and Cell Line Irradiation for Clonogenic Survival. A total of 5×10^5 cells were plated into sterile T25 flasks (Falcon Plastics, Lincoln Park, NH) and, typically, 2×10^6 cells were available for transfection in each flask after 48 hours. Virus was diluted in serum-free DMEM/F12 until ready for transfection. The cells in each flask were washed in phosphate buffered solution to remove any residual serum, which might bind with the virus and decrease the MOI. The viral solution (1 ml) was then gently placed onto the monolayer. The flasks were then returned to the incubator for a total of one hour. At 10-minute intervals, the flasks were gently rocked to insure even mechanical distribution of the viral solution over the cells. Control flasks, with and without control vector, were exposed to identical manipulations during this process. After one hour in the incubator, 4 ml of complete medium, with serum, was added to each flask. This effectively ended the transfection process. The flasks were then returned to a dedicated incubator for future planned irradiation. Forty-eight hours after viral transfection, flasks were removed from the incubator and placed on ice for 20 minutes. Flasks were then irradiated with a high dose-rate Nasatron cesium unit. Immediately after irradiation, flasks were trypsinized, serial dilutions performed, and known numbers replated onto 100 mm dishes in triplicate for all dose irradiation dose levels, with and without virus. Approximately 12 days were needed to allow for macroscopic colony formation. The colonies were stained with gentian violet, and counted. The surviving fraction relative to the unirradiated cells was then calculated. Triplicate determinations of each radiation dose and dilution was performed in every experiment

and an intra-experiment average calculated. The points shown on the clonogenic survival curves are the inter-experiment averages calculated from the intra-experiment averages.

TUNEL Staining. Apoptosis was measured using a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. The procedure involved culturing, fixing, and staining the cells directly on glass slides. The slides were prepared for cell culture by marking a 1.5 – 2.0 cm circle with a hydrophobic slide marker (Research Products International, Mount Prospects, IL), cleaning with 70% ethanol for 30 min, and eliminating possible contaminating viruses by UV lamp exposure (30 W) for 30 min at a 20 cm distance. The sterile slides were placed in a 100 mm cell culture dish and 150 µl of culture medium with the cells (3×10^4 for PC3 and 7×10^4 for LNCaP) was placed into the center of each slide. The cells were cultured for 24 hr and an additional 15 ml was added to the plates. The cells were cultured for another 2 – 4 days, until an even distribution of nonconfluent cells was present. The cells on the slide monolayers were counted and then treated by first rinsing in 15 ml of PBS and adding 100 µl of viral suspension at the desired MOI in serum-free medium. The cells were incubated for 1 hr at 37° C and 100 ml of complete medium with serum added. The cells were further incubated another 24 hr before irradiation with 2 or 8 Gy given in a single fraction. After removing the medium, the cells fixed in 4% formaldehyde for 30 min. The formaldehyde was then removed and the slides placed in 70% ethanol. The TUNEL staining was done within a few days of fixation.

The Apop Tag kit (Oncor, Gaithersburg, MD) was used for TUNEL staining. In this method 3'-OH ends of DNA are tailed with digoxigen-nucleotide (using terminal deoxynucleotidyl transferase or TdT). The complex is then bound with anti-digoxigen antibody

peroxidase conjugate and stained with DAB, which provides a dark brown color. (15). The apoptotic index was calculated by dividing the number of apoptotic cells by the cell population (16). The cells were counterstained with hematoxylin and eosin. Positive controls were included with each group of samples stained.

RESULTS

Radiosensitization of LNCaP cells using Ad5-p53. Eleven control experiments were performed to accurately characterize the clonogenic response of LNCaP cells to irradiation without the presence of viral vector. Four experiments were then performed with Ad5-p53 plus radiation. An MOI of 40-70 was selected based on preliminary data that demonstrated approximately 50% transfection efficiency. Treatment with Ad5-p53 reduced control plating efficiency from 42% to 1.5% in the LNCaP cell line. Normalizing to plating efficiency, the surviving fraction after 2 Gy of gamma-irradiation was 19.7% without Ad5-p53 compared to 6.3% with vector (Figure 1). Similarly, survival after 4 Gy was reduced from 1.1% to 0.23% with Ad5-p53 exposure.

In order to demonstrate that the radiosensitization was due to the presence of p53 in the viral vector, rather than nonspecific effects of the vector itself, experiments were performed using Ad5- β gal. Results demonstrated that Ad5- β gal had no significant radiosensitization when transfected into the LNCaP cell line (Figure 2).

Radiosensitization of PC3 cells using Ad5-p53. Very reproducible clonogenic cell survival results were observed in PC-3 cells irradiated in the absence of virus. The PC-3 cell line was quite susceptible to Ad5-p53 alone, exhibiting a reduction in control plating efficiency from 88.6% to 0.55% (Figure 3). The surviving fraction after adjusting for plating efficiency was 60.3% at 2 Gy without vector and 36.9% with the addition of Ad5-p53 at 40 MOI. At 4 Gy, the surviving fraction decreased from 24.6% without virus to 0.5% with transfection. These differences were statistically significant ($p<0.05$, student's t-test).

Apoptotic response to Ad5-p53 plus 2 Gy radiation. The in vitro apoptotic response of LNCaP and PC3 exposed to Ad5-p53 plus radiation is shown in Table 1. The results show a dramatic increase in apoptosis in both cell lines when this combination was used as compared to the controls. Apoptosis appears to be a significant mechanism of cell death when prostate cancer cells are treated with Ad5-p53 plus irradiation.

DISCUSSION

Radiotherapy is the most common treatment for high risk prostate cancer. Through the use of PSA to monitor the efficacy of treatment, it has become apparent that few high risk patients are cured with radiotherapy alone (17, 18). The results with radical prostatectomy are probably worse (19,20). While a proportion of such high risk patients fail distantly early after the onset of a rising PSA, there is evidence that the main site of initial failure is local (21). Consequently, novel techniques for radiosensitization hold promise for improving the cure fraction. One strategy is combining androgen ablation with radiation. Preliminary evidence indicate radiosensitization occurs when androgen ablation and radiation are applied in a certain sequence (2). However, the clinical trials that have been published to date (22, 23) do not sort out the advantage of androgen ablation plus radiation over androgen ablation alone, making conclusions of real benefit of the combination unclear. Even if androgen does sensitize prostate cancer cells to radiation, there is a need to develop new methods of radiosensitization based on molecular mechanisms. Our approach has been to alter the intracellular milieu on a molecular level such that cell death via apoptosis is the favored pathway with exposure to radiation.

The p53 gene product is prototypal of a gene therapy target that is integral to the apoptotic response to irradiation. When a tumor cell expresses mutated p53 or does not express p53 at all, replacement with p53 gene therapy has resulted in radiosensitization (14,24,25). What was uncertain, was whether p53 transgene expression would radiosensitize cells that express wild type p53. Since most prostate cancers express wild type p53, this was a key question of the research performed here.

The data presented here show that Ad5-p53 transfection into cultured human prostate adenocarcinoma cells results in sensitization to irradiation. Radiosensitization was evident using clonogenic survival and apoptosis assays. This effect was observed both in the p53^{wildtype} LNCaP cell line, as well as the p53^{null} PC-3 cell line. Thus, even prostate cancer cells with p53^{wildtype} expression were radiosensitized, albeit to a lesser degree than p53^{null} cells (see Figures 1 and 3).

The mechanism of this toxicity is not exclusively due to the restoration of p53 function, since LNCaP cells express wild-type p53. We have also observed that p53 expression in LNCaP cells is enhanced within hours of irradiation (data not shown), typical of other cell lines with functional p53. The data indicate that even in the presence of wild-type p53, the induction of p53 transgene overexpression by Ad5-p53 is enough to promote apoptosis as the preferred response after irradiation. The greatest radiosensitization by Ad5-p53, however, was seen with p53 replacement in p53^{null} PC3 cells; PC3 cells were radiosensitized to a greater degree than p53^{wildtype} LNCaP cells. The observation of supra-additive toxicity in both cell lines attests to the potential application of Ad5-p53 for the sensitization of prostate cancer cells to radiation.

In conclusion, the use of p53 gene therapy has made it possible to preferentially induce cell death to irradiation rather than transient G1 arrest and unwanted repair. Localized high risk prostate cancer has many potential advantages as a model for the use of gene therapy (26). Prostate cancer has a long natural history, which ameliorates some of the concerns of an inefficient delivery system by virtue of the fact that repeated applications are possible and anatomically the prostate is relatively easy to access via transperineal injection of gene vector delivery systems. With the advent of PSA as both a screening and follow-up endpoint, it has become clear that the eradication of prostate cancer is more difficult than was previously believed. Using the pretreatment prognostic factors of PSA, Gleason score, and palpitory stage,

patients at high risk of failing radiotherapy alone may be identified and targeted using gene therapy techniques.

Table 1. Apoptotic response of LNCaP and PC3 cells to Ad5-p53 plus 2 Gy radiation using the TUNEL assay.

<u>Vector</u>	<u>2 Gy RT</u>	Apoptotic Index*	
		<u>LNCaP</u>	<u>PC3</u>
No	No	0	0.03
No	Yes	0	0.65
Ad5-pA [†]	No	0.01	0
Ad5-pA [†]	Yes	0.26	9.35
Ad5-p53 [‡]	No	1.38	1.43
Ad5-p53 [‡]	Yes	30.36	18.2

* Representative experiment of two; 2000 cells counted per group.

[†] Used at 50 MOI.

[‡] Used at 25 MOI.

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Figure Legends

Figure 1. Clonogenic surviving fraction of LNCaP cells incubated for 1 hour with (solid circles) and without Ad5-p53 (open squares) and irradiated 24 hours later. The error bars represent one standard deviation above and below the mean.

Figure 2. Clonogenic surviving fraction of LNCaP cells incubated for 1 hour with (solid circles) and without Ad5- β Gal (open squares) and irradiated 24 hours later. The error bars represent one standard deviation above and below the mean.

Figure 3. Clonogenic surviving fraction of PC3 cells incubated for 1 hour with (solid circles) and without Ad5-p53 (open squares) and irradiated 24 hours later. The error bars represent one standard deviation above and below the mean.

Figure 3. Clonogenic surviving fraction of PC3 cells incubated for 1 hour with (solid circles) and without Ad5- β Gal (open squares) and irradiated 24 hours later. The error bars represent one standard deviation above and below the mean.

Figure 1, Colletier et al

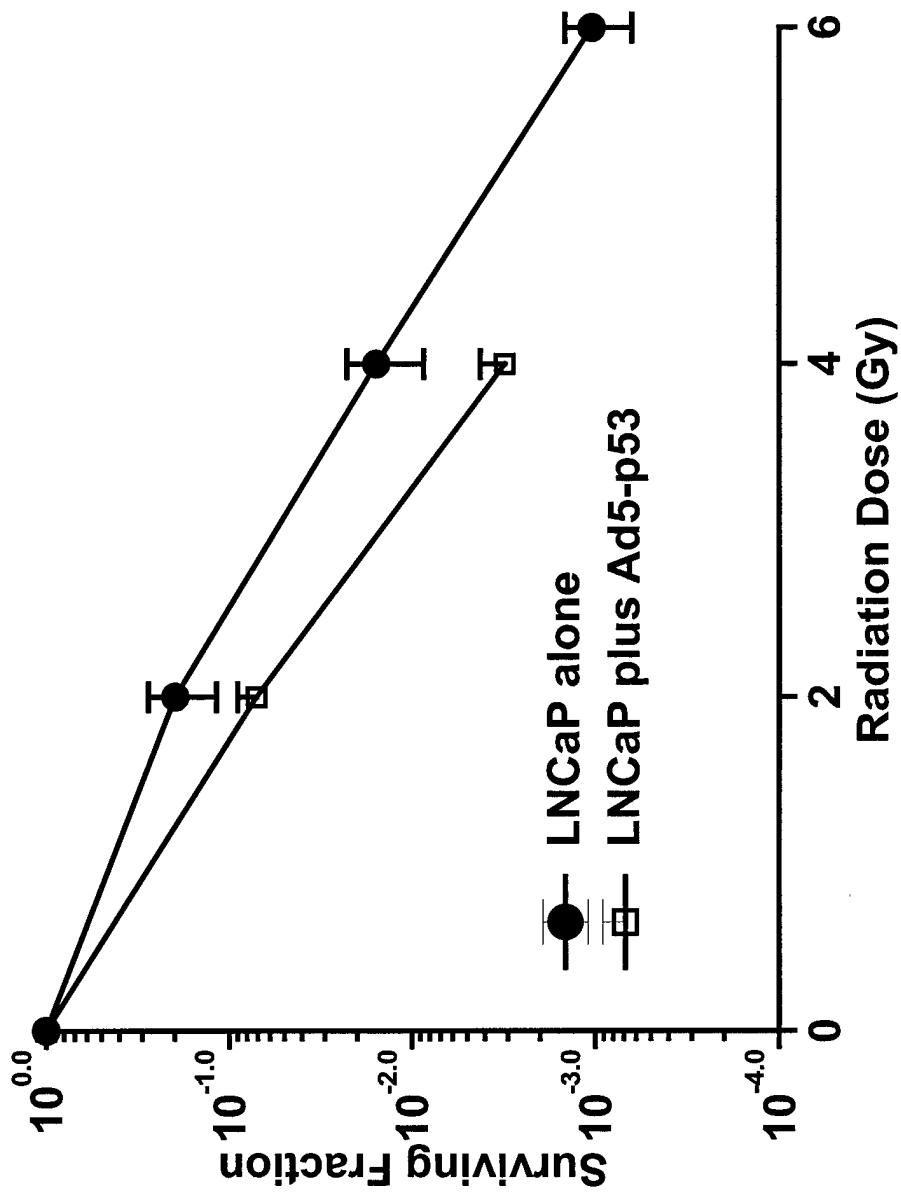


Figure 2, Colletier et al

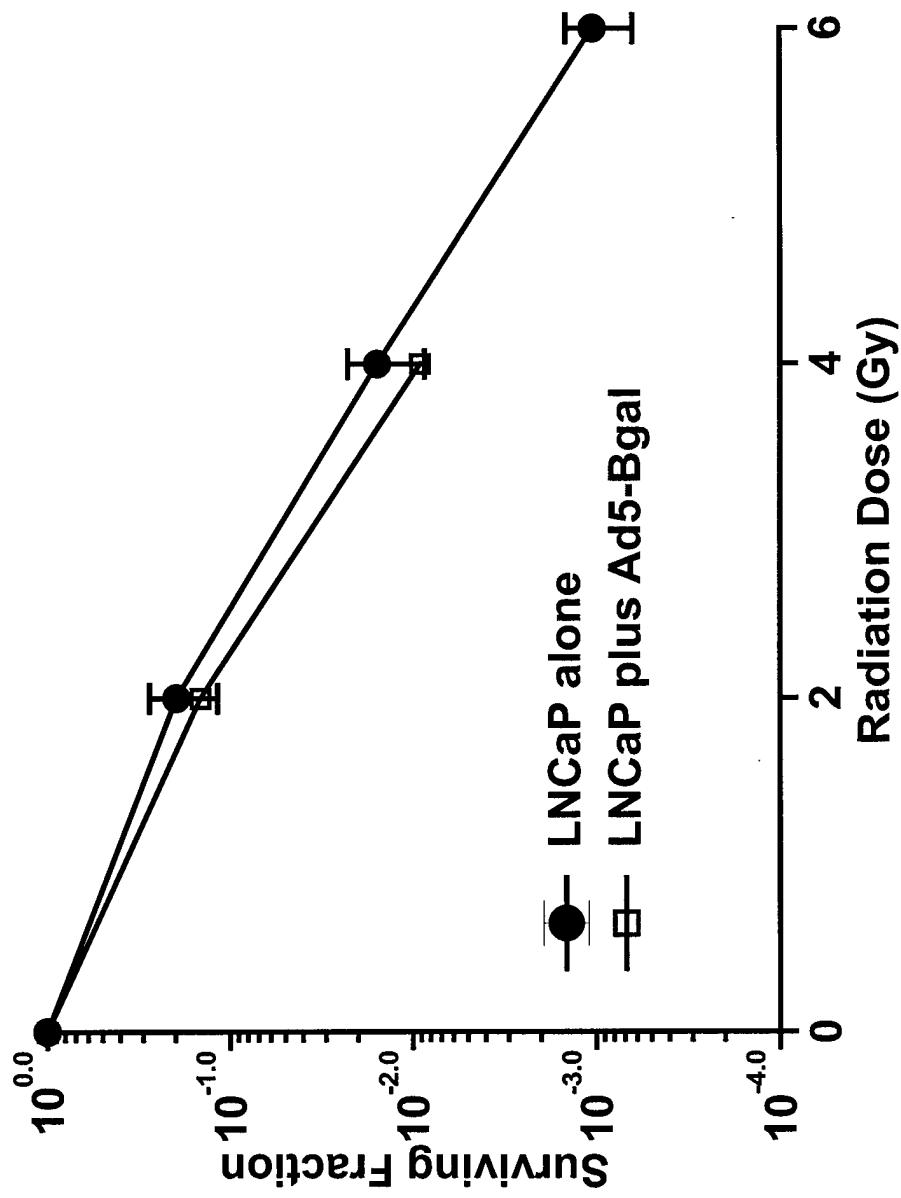


Figure 3, Colletier et al

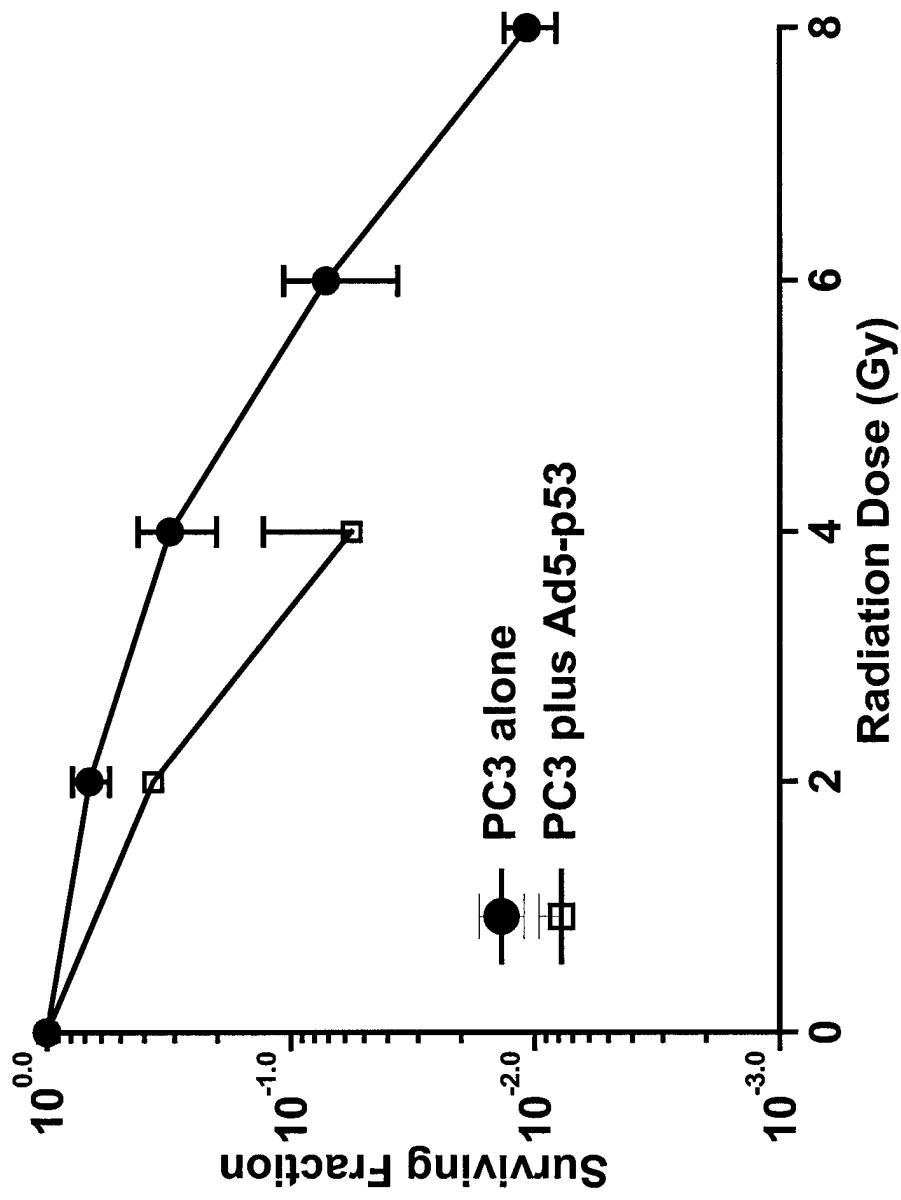
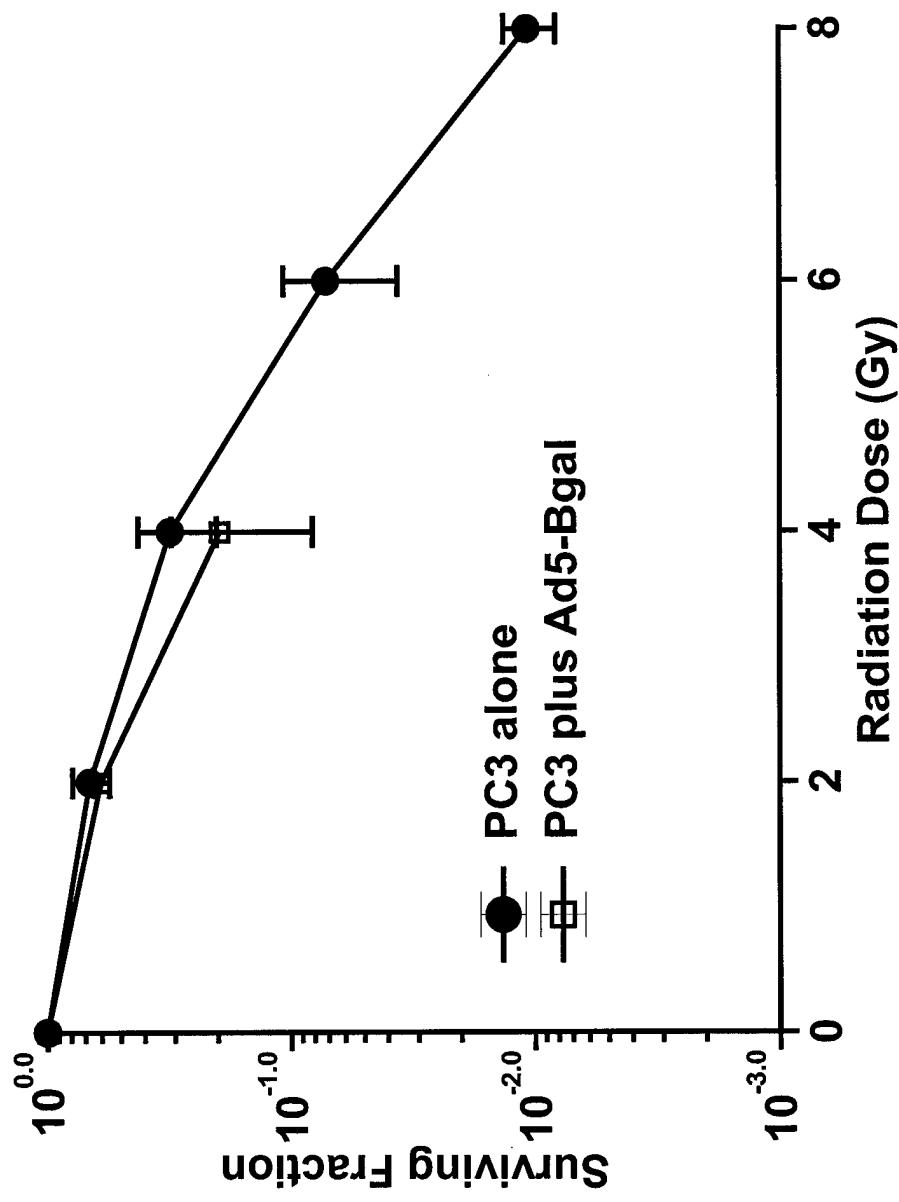


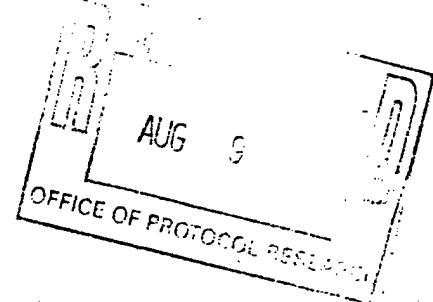
Figure 4, Colletier et al



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August 5, 1999



MEMORANDUM

TO: Cathy Scherer, IRB Coordinator
Office of Protocol Research (Box 38)

FROM: Alan Pollack, M.D., Ph.D. *AP*
Associate Professor

SUBJECT: Contingent Approval of Protocol ID99-205 entitled "A Randomized Phase II Study of Adenovirus-p53 Plus Radioactive Seed Implant Versus Seed Implant Alone for PSA Relapse After External Beam Radiotherapy"

The amendments have been made in the *Informed Consent*, adding surgery as an alternative procedure. If you have any questions, please contact my office.

AP:bkb

ok *PL* *8-13-99*
GABRIEL LOPEZ-BERESTEIN, M.D.

THE UNIVERSITY OF TEXAS M.D. ANDERSON CANCER CENTER
DEPARTMENT OF RADIOTHERAPY

A RANDOMIZED PHASE II STUDY OF ADENOVIRUS-p53 PLUS RADIOACTIVE
SEED IMPLANT VERSUS SEED IMPLANT ALONE FOR PSA RELAPSE AFTER
EXTERNAL BEAM RADIOTHERAPY

Abstract

Eligibility Checklist

- 1.0 Objectives
- 2.0 Background
- 3.0 Drug Information
- 4.0 Patient Eligibility
- 5.0 Treatment Plan
- 6.0 Pretreatment and Treatment Evaluations
- 7.0 Post-treatment Evaluation
- 8.0 Data Collection
- 9.0 Reporting of Adverse Reactions
- 10.0 Statistical Considerations
- 11.0 References

Appendix A: Tests and Schedules

Appendix B: QOL Questionnaires

Appendix C: Zubrod Performance Status Scale

Appendix D: Clinical Staging

Appendix E: Acute Toxicity Grading

Appendix F: Late Toxicity Grading

Appendix G: Reporting of Adverse Events

Appendix H: Temperature Log

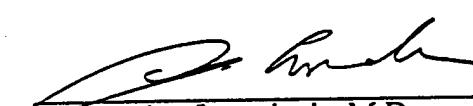
Consent

PRINCIPAL INVESTIGATOR:

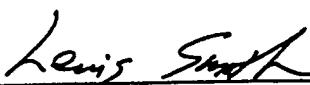


Alan Pollack, M.D., Ph.D.
Department of Radiation Oncology

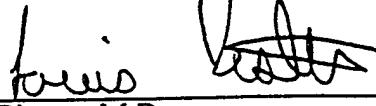
COLLABORATORS:



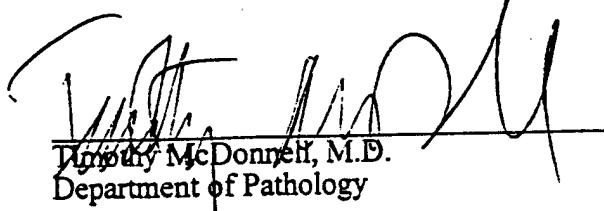
Christopher Logothetis, M.D.
Department of GU Medical Oncology



Lewis Smith, M.D.
Department of Radiation Oncology



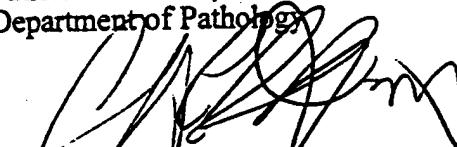
Louis Pisters, M.D.
Department of Urology



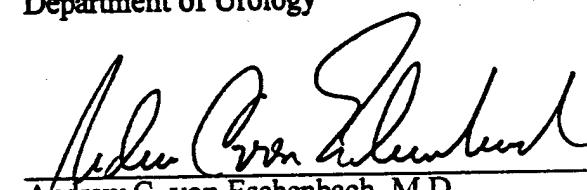
Timothy McDonnell, M.D.
Department of Pathology



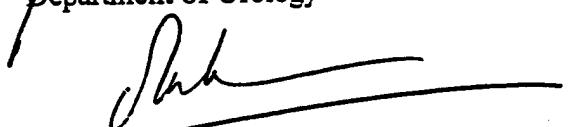
Patricia Troncoso, M.D.
Department of Pathology



Curtis Pettaway, M.D.
Department of Urology



Andrew C. von Eschenbach, M.D.
Department of Urology



Kim-Anh Do, Ph.D.
Department of Biomathematics

Eligibility Checklist

- (Y) Prior external beam radiotherapy of the prostate for clinical Stage T1-T3 (Nx, M0) adenocarcinoma of the prostate.
- (Y) No prior radical prostate surgery.
- (Y) Evidence of a rising PSA (3 consecutive rises on followup) after external beam treatment.
- (Y) PSA doubling time of >1 year. Calculated PSA doubling time: _____ months.
- (Y) No evidence of metastases by bone scan within 1 mo of enrollment..
- (Y) No enlarged adenopathy on CT-scan of the pelvis within 1 month of enrollment.
- (Y) No palpable evidence of palpable extraprostatic tumor extension on rectal exam.
- (Y) PSA ≤10. Current PSA: _____ ng/ml done within two weeks of enrollment.
- (Y) Prostate volume by ultrasound is ≤65 cm³. Current prostate volume by ultrasound: _____ done within 1 month of enrollment.
- (N) Androgen ablation, except for below.
 (Y) May be have been used for ≤6 months, but must have been stopped for >1 year prior to study enrollment.
- (Y) Zubrod Performance Status ≤2.
- (Y) No history of grade 3 late radiation reaction from external beam radiotherapy.
- (Y) No history of HIV positivity or chronic hepatitis B or C infections.
- (Y) No history of steroid medications of greater than two months duration. (Such patients are considered to have been treated with hormonal therapy).

PROTOCOL ABSTRACT**PROTOCOL: (Abbreviated title) (2 lines/75 characters per line)(USE 12 PT ONLY)**

A Randomized Phase II Study of Adenovirus-p53 Plus Radioactive Seed Implant Versus Seed Implant Alone For PSA Relapse After External Beam Radiotherapy For Prostate Cancer

STUDY CHAIRMAN: Alan Pollack, M.D., Ph.D.**OBJECTIVES:**

1. To determine the feasibility and toxicity of administering wild type p53 in an adenovirus (Ad5-p53) vector plus salvage I-125 seed implantation, as compared to I-125 seed implant alone, for patients with biochemical failure after external beam radiotherapy.
2. To examine the differences in molecular response in the two treatment arms in terms of pathological (necrosis by H&E staining and apoptosis by TUNEL assay), proliferation (Ki-67/MIB-1), and molecular (p53 and bcl-2) parameters.
3. To assess response to the treatments via prostate biopsy at 1 and 2 years (pathologic response), nadir PSA level with 2 years minimum followup (biochemical response), and clinical response (palpable response). The main response parameter will be prostate biopsy at 1 year.

RATIONALE: (Be as concise as possible)

A rising PSA after external beam radiotherapy for patients with clinically localized prostate cancer is usually representative of local persistence of disease. Those with a negative metastatic work-up are left with the main options of observation, androgen ablation alone, salvage prostatectomy, or salvage brachytherapy. The first two options are palliative. Salvage prostatectomy is associated with considerable morbidity, although 30-50% may be salvaged. Salvage brachytherapy appears to be associated with less morbidity and salvage rates of about 35%. The rationale is to sensitize the prostate cancer cells to radiation by injecting Ad5-p53 directly into the prostate, allowing for enhanced cure rates using lower seed implant radiation doses. This strategy should reduce morbidity and enhance efficacy over salvage seed implant monotherapy.

ELIGIBILITY: (List Major Criteria)

- 1) Prior external beam radiotherapy for clinical stage T1-T3 (Nx, M0) adenocarcinoma of the prostate.
- 2) Evidence of a rising PSA (3 consecutive rises on followup).
- 3) The PSA doubling time should be greater than 1 year.
- 4) A positive post-external beam radiotherapy prostate biopsy for adenocarcinoma.
- 5) No evidence of metastases by bone scan and CT-scan of the pelvis.
- 6) No evidence of palpable extraprostatic extension at the time of enrollment.
- 7) PSA \leq 10 ng/ml.
- 8) Prostate volume \leq 55 cc.
- 9) Androgen ablation is permitted if it was for \leq 6 mo and was stopped over 1 year prior to enrollment.
- 10) Zubrod Performance Status \leq 2.
- 11) No history of grade 3 radiation reaction to external beam radiotherapy
- 12) No history of HIV positivity or chronic hepatitis B or C infections.

- 13) No history of steroid medications of greater than two months duration. (Such patients are considered to have been treated with hormonal therapy).

TREATMENT PLAN:

Patients with a rising PSA post-external beam radiotherapy will be randomized between Ad5-p53 gene therapy using INGN 201 plus I-125 seed implant and I-125 seed implant alone. Stratification will be based on whether androgen ablation was given prior to the implant and whether the pre-implant PSA was ≤ 2 ng/ml or above. The I-125 seed implant dose will be 110 Gy specified to a planning target volume of 2 – 5 mm around the prostate. Ad5-p53 gene therapy will be administered in three intraprostatic injections at 3×10^{12} viral particles per injection. The first injection of INGN 201 will be at the time of the I-125 implant and the second and third injections at two and four weeks thereafter.

STATISTICAL CONSIDERATIONS:

A total of 74 patients will be entered; 37 per arm. The primary objective of this study is to assess efficacy in terms of pathologic response by prostate biopsy at 1 year and toxicity in terms of bladder and rectal late morbidity \geq grade 3 for Ad5-p53 gene therapy plus radioactive I-125 seed implant versus seed implant alone in patients refractory to external beam radiotherapy. From the sample of 37 patients for each arm, if (a) there are 14 or fewer responses for the combined treatment arm, then the treatment is rejected due to inadequate response; (b) if there are 9 or more adverse events then the treatment is rejected due to excessive toxicities; (c) if there are more than 14 responses and fewer than 10 adverse events then the treatment is recommended for further consideration. With the above rules for the combined treatment arm, the overall type I errors are 2% (poor response and excessive toxicity), 10% (poor response and acceptable toxicity), 14% (good response and excessive toxicity), and a type II error will be 14%. Interim analysis for possible early trial termination will be performed after 15 patients in each arm have been enrolled. The purpose of the randomization is to provide unbiased estimators of the effects of adding gene therapy on the variables of interest.

PATIENT EVALUATION: (Pretreatment and Interim Testing)

All patients must have a post-external beam radiotherapy prostate biopsy, pre-seed implant prostate specific antigen (PSA), complete blood count with differential and platelets, PT, PTT, prostate ultrasound volume, chest x-ray, bone scan, and pelvic CT-scan before study enrollment. Prostate biopsies will be obtained at 1 day and again at 2 years after the first p53 injection. The patient will undergo history and physical examination post-implant at 2 weeks, one month, every 6 months for two years, and every 12 months thereafter. Serum PSA will be drawn post-implant at 2 weeks, one month, every 3 months for two years, and every 6 months thereafter.

ESTIMATED ACCRUAL: 74

IT IS ESTIMATED THAT ACCRUAL WILL BE 2 PARTICIPANTS PER MONTH.

SITE OF STUDY: (PLEASE CIRCLE THE APPROPRIATE ANSWER)

THIS PROTOCOL IS PERFORMED ON AN:

INPATIENT

OUTPATIENT

BOTH

LENGTH OF STAY: (WHAT IS THE LENGTH & FREQUENCY OF HOSPITALIZATION)

N/A

RETURN VISITS: (HOW OFTEN MUST PARTICIPANTS COME TO MDACC)

2 weeks, 1 month, every 6 months for 2 years and then annually.

HOME CARE: (SPECIFY WHAT (IF ANY) TREATMENT MAY BE GIVEN AT HOME)

None

WHERE WILL STUDY BE CONDUCTED:

A) ONLY AT MDACC

B) MDACC + COMMUNITY PROGRAMS

C) INDEPENDENT MULTICENTER

(CCOP, NETWORK)

ARRANGEMENTS

NAME OF SPONSOR/FUNDING SOURCE:

Cost to patient: The first Ad5-p53 injection will be done at the time of the implant and will not be associated with additional cost to the patient. The second and third p53 injections will be administered under conscious sedation and will cost approximately \$2,000 per injections. These costs will be defrayed partly by the GU center at MDACC, Introgen Therapeutics, Houston, Texas, and Amersham Corporation. The implant will cost approximately \$22,500.

COMPETING PROTOCOLS: (Protocol Number (s))

N/A

NAME OF RESEARCH NURSE/DATA MANAGER RESPONSIBLE FOR PROTOCOL:

Joy Phillips, R.N.

SUBMIT PROTOCOL TO CLINICAL RESEARCH CENTER REVIEW COMMITTEE:
YES NO

IF YOUR PROTOCOL HAS A DIAGNOSTIC STEP REQUIRING INFORMED CONSENT AND REGISTRATION ON THE PROTOCOL (E.G., A BLOOD TEST OR BIOPSY) THAT WILL DETERMINE WHETHER OR NOT THE PATIENT WILL SUBSEQUENTLY RECEIVE OR NOT RECEIVE EXPERIMENTAL THERAPY. PLEASE CHECK THE APPROPRIATE BOX(ES) SO THAT THE APPROPRIATE FIELDS MAYBE ESTABLISHED IN PDMS:

BLOOD TEST	YES <input type="checkbox"/> NO <input type="checkbox"/>	BIOPSY	YES <input type="checkbox"/> NO <input type="checkbox"/>	OTHER	YES <input type="checkbox"/> No <input type="checkbox"/>
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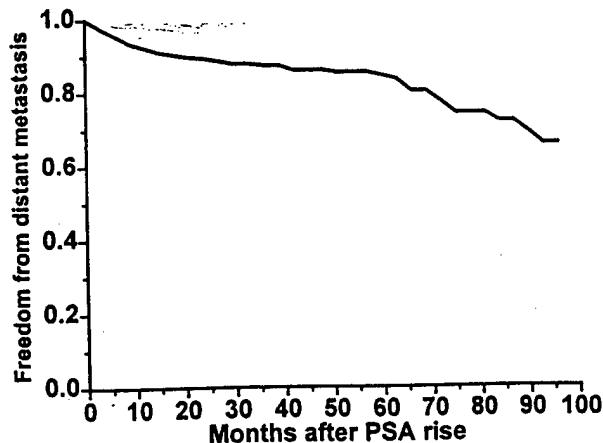
1.0 OBJECTIVES

- 1.1 To determine the feasibility and toxicity of administering wild type p53 in an adenovirus (AD-p53) vector plus salvage I-125 seed implantation, as compared to I-125 seed implant alone, for patients with biochemical failure after external beam radiotherapy.
- 1.2 To examine the differences in molecular response in the two treatment arms in terms of pathological (necrosis by H&E staining and apoptosis by TUNEL assay), proliferation (Ki-67/MIB-1), and molecular (p53 and bcl-2) parameters.
- 1.3 To assess response to the treatments via prostate biopsy at 1 and 2 years (pathologic response), nadir PSA levels with 2 years minimum followup (biochemical response), and clinical response (palpable response). The main response parameter will be prostate biopsy at 1 year.

2.0 BACKGROUND

- 2.1 Failure patterns after external beam radiotherapy. The rising PSA profile after external beam radiotherapy or surgery is a harbinger of disease relapse. Biochemical failure precedes clinically detectable disease relapse by 3-5 years on average (Pollack et al, 1994) and a number of studies show that it is a correlate of distant metastasis. From our database of 1160 men with stage T1-T4, Nx/N0, M0 prostate cancer treated solely with external beam radiotherapy, every case of distant relapse was preceded by biochemical relapse (Smith et al, in press). In the majority of cases with isolated biochemical failure, local tumor persistence is documented on prostate biopsy as the only initial evidence of disease. For the 341 men with evidence of a rising PSA, the distant metastasis rate was 34% at 8 yr (calculated from the time of a rising PSA; Figure 1). The PSA doubling time after treatment, be it external beam radiotherapy (Smith et al, In press) or surgery (Pound et al, 1999), correlates with the development of distant metastasis. A PSA doubling time of <1 year was associated with an 8 yr actuarial rate of distant metastasis of 53%, as compared to 17% when the doubling time was >1 yr. The inherently long lag-time between biochemical and distant failure, local persistence as the main site of failure, and ability to select patients with a low risk of developing distant metastasis based on PSA doubling time suggests that many of these patients might be salvaged with aggressive local treatment.

Figure 1. Freedom from distant metastasis in patients with a rising PSA after external beam radiotherapy



2.2 Salvage therapy. External beam radiotherapy is a common treatment for prostate cancer and has proven to be efficacious. However, local recurrence, documented by prostate biopsy, is seen in over 30% and represents the most common site of first failure. The salvage of patients with local recurrence after external beam radiotherapy (EBR) has in general been poor. In selected patients with delayed recurrence >2 yr after EBR and in whom the PSA is <10 ng/ml, salvage radical prostatectomy appears to control about 45% for 5 yr (Amling et al, 1999; Gheiler et al, 1998; Bochner et al, 1998; Roger et al, 1995; Pontes et al, 1993). However, the morbidity of this approach is quite high, particularly for men >70 years old. An alternative that is gaining favor is salvage implant monotherapy using Iodine-125 or palladium-103 (Grado et al, 1999; Butler et al, 1997; Dattoli et al, 1997; Cumes et al, 1981; Goffinet et al, 1980). The problem with re-irradiating the prostate with brachytherapy is that the dose used is often limited for fear of sequelae. The more recent ultrasound-based studies (Grado et al, 1999; Butler et al, 1997; Dattoli et al, 1997) have demonstrated reasonable complication rates with biochemical control (freedom from a rising PSA) seen in about 35%. *The key question posed is whether radiosensitization using p53 gene therapy is feasible and of low morbidity, and whether the tumor is completely eradicated from the prostate using this strategy.* This key question will be addressed via a randomized Phase II trial comparing radioactive I-125 seed implant monotherapy with Ad5-p53 gene therapy and seed implant.

There are a small number of studies with limited patient numbers (Grado et al, 1999; Butler et al, 1997; Dattoli et al, 1997; Cumes et al, 1981; Goffinet et al, 1980) in which brachytherapy has been explored as salvage for local disease persistence after external beam radiotherapy. Salvage brachytherapy is an old concept (Cumes et al, 1981; Goffinet et al, 1980) that has recently been improved upon through the use of ultrasound guidance of seed placement. There are now a number of groups with experience, even though much of the available data is immature and has not been formally published. Drs Stock and Stone (1998) have a large experience in the treatment of prostate cancer with brachytherapy and have treated over 100 patients using Palladium-103 for external beam failures (personal communication). Using a prescribed dose of 90 Gy they have noted few cases of incontinence and no rectal complications. Drs. Beyer and Preistly (1997) have used Iodine-125 for salvage in over 50 patients with local progression after external beam radiotherapy to a prescribed dose of 112 Gy. They found a 25% incontinence rate (personal communication). The largest published experience in the ultrasound era is by Grado et al (1999) wherein 49 patients were treated with full dose Palladium-103 (120 Gy) or Iodine-125 (160 Gy). Actuarial 3 and 5 year disease-specific survival was 89% and 79%, and biochemical control was 48% and 34%. No serious complications were observed.

2.3 Gene therapy studies in model systems. Ad5-p53 is the prototypal gene therapy vector. p53 transgene expression has been shown to radiosensitize colon cancer cells in vitro and in vivo (Spitz et al, 1997), and has been used in lung cancer patients on protocol as a radiosensitizer (personal communication, Jack Roth, M.D.). Our in vitro and in vivo experiments have confirmed that Ad5-p53 sensitizes using human prostate cancer cells to radiation. The model systems used were the p53^{wildtype} LNCaP and p53^{null} PC3 lines. The exposure of either of these cell lines in vitro with Ad5-p53 (multiplicity of infection of 40-70) 24 hr prior to irradiation at single fraction doses of 2-6 Gy caused significantly reduced clonogenicity, as compared to control vectors (Ad5-βGal or Ad5-polyadenylation sequence [Ad5-pA]) plus radiation or radiation alone (Collatier et al, 1998). The enhanced cell killing from Ad5-p53 plus radiation was clearly supra-additive (Figure 2).

Figure 2A: Effect of Ad5-p53 + RT on PC3

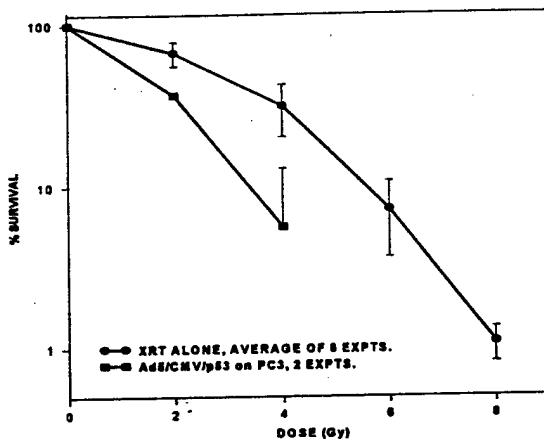
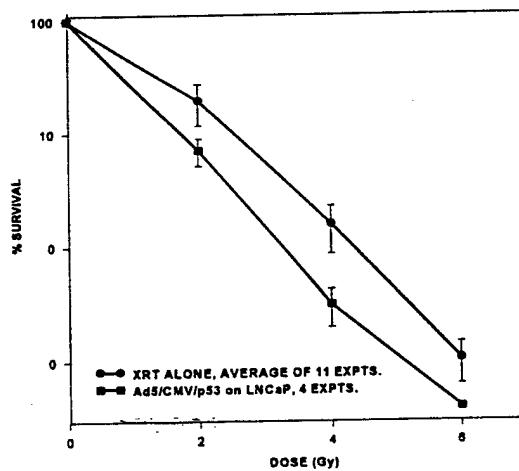


Figure 2B: Effect of Ad5-p53 + RT on LNCaP



In vivo experiments in nude mice using PC3 involved the implantation of 2×10^6 cells subcutaneously in a hind leg and beginning treatment at a tumor volume of 200 mm^3 . Three intratumoral injections (days 1, 4, and 7) were given with 3×10^8 plaque forming units, followed by 5 Gy pelvic irradiation in one fraction using a cesium source. Tumor volume measurements were obtained three days per week. The PC3 tumor volume growth curves were log-transformed, and fitted using linear regression. The times (in days) for the tumors to reach 500 mm^3 were calculated as 10.7 ± 0.7 for the saline control (no virus), 9.8 ± 2.1 for Ad5-pA (virus control), 15.6 ± 1.6 for Ad5-p53, 14.6 ± 1.5 RT (5 Gy), 14.6 ± 1.5 for Ad5-pA plus RT, and 31.3 ± 5.3 for Ad5-p53 plus RT. The latter group times were significantly different at $p < 0.05$ (one way ANOVA, Scheffe test) from all of the other groups. The absolute delay in

tumor growth to 500 mm³ relative to the saline control was used to calculate the enhancement factor [Abs delay(Ad5-p53+RT - Ad5-p53)/RT], which was 4.0. The Ad5-pA controls were not included in the calculation because no significant delays were seen.

LNCaP cells (2×10^6 in 24 μ l) were injected orthotopically into the prostates of nude mice and tumor weight approximated using serum PSA obtained from weekly tail vein bleedings. There is a linear relationship between tumor weight and serum PSA; linear regression results revealed that tumor weights of 7, 10, and 20 mg correlated with PSAs of 1.4, 3.0, and 8.5 ng/ml. The target PSA for the studies was 5 ng/ml. The animals were then anesthetized, the prostate surgically exposed, and 4.5×10^8 pfu injected in 24 μ l. The intraprostatic injections were done twice (days 1 and 4), and 5 Gy pelvic irradiation was administered 24 hr later (Day 5). LNCaP tumor growth was determined via weekly serum PSA measurements, and a serum PSA of >1 ng/ml six weeks after treatment was considered evidence of regrowth. There were five animals per group. The number of animals with evidence of regrowth were 5 for the saline control, 5 for Ad5-pA, 4 for Ad5-p53, 4 for RT alone (5 Gy), 4 for Ad5-pA + RT, and 1 for Ad5-p53 + RT. These results are also consistent with a supra-additive inhibition of tumor growth.

- 2.4 Ad5-p53 gene therapy studies in prostate cancer patients. There is substantial evidence that Ad5-p53 sensitizes both p53^{wildtype} and p53^{null} prostate cancer cells. The technique of intraprostatic injection has already been devised and the effect of p53 transgene expression has been documented. A protocol examining the effects of injecting Ad5-p53 prior to radical prostatectomy has recently been completed. There were 30 patients that were enrolled and 26 received escalating doses of Ad5-p53 from 3×10^{10} to 3×10^{12} viral particles per injection for 3 injections. The preliminary data show an increase in p53 expression and apoptosis 24 hr following injection. Low grade fever was the most common side effect. No significant and measurable increase in surgical or other toxicity was encountered at the highest dose level (Tables 1). This is the dose level that will be used in the current trial.

Table 1: Ad5-p53 Plus Prostatectomy Side Effect Events

Number of Patients with

Event	Grade 1	Grade 2	Grade 3	Grade 4
Perineal Pain	12	10	0	0
Fever	11	19	0	0
Chills	6	5	0	0
Headache	2	2	0	0
Hematospermia	0	3	0	0
Hematuria	1	1	0	0
Scrotal Edema	1	2	0	0

Preliminary evidence for antitumor activity existed by many parameters: Regression of prostate cancer by 25% occurred within six weeks in 7/26 available for analysis at this time. In addition, the serum PSA (Fig 3) concentration declined in the majority of patients (data not shown). The decline in the serum PSA concentration occurred in most patients despite repeated puncture of the prostate which frequently results in a substantial rise in the PSA concentration. Biopsies at 24 hr after p53 injection showed enhanced apoptosis (Table 3). The pathological findings from radical prostatectomy at revealed that the patients had high grade cancer which was regionally advanced, which suggests that p53 therapy alone is insufficient for patient in this category and that patients with poor prognostic features were reliably selected.

Figure 3. Changes in tumor volume from preoperative Ad5-p53.

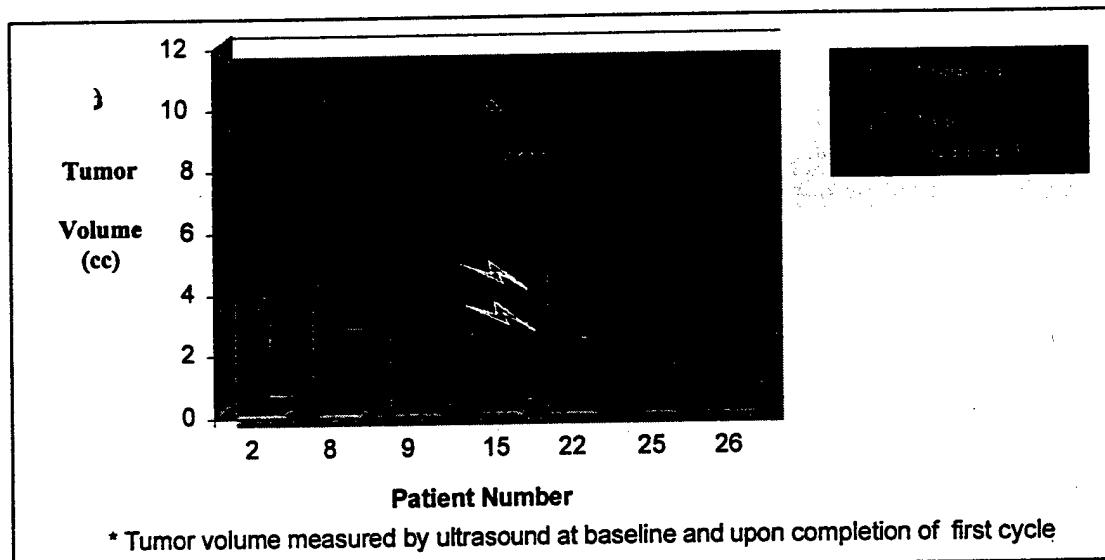


Table 3. The induction of apoptosis 24 hr after Ad5-p53 treatment.

Pt #	p 5 3		A p o p t o t i c I n d e x	
	Baseline	Day 1	Baseline	Day 1
14	-	+	0.4 %	0.8 %
17	-	+	0.6 %	2.75 %
18	-	+	0.6 %	0.5 %
19	-	+	0.5 %	1.6 %
20	-	-/+	0.3 %	2.7 %
21	-	++	0.8 %	4.6 %
23	+	-/+	1.13 %	0.6 %
24	-	-/+	0.4 %	1.1 %

These are the first clinical data demonstrating that p53 transgene expression results in increased programmed cell death in human prostate cancer.

- 2.5 Quality of life, Prostate Cancer Late Side Effect, and AUA symptom score questionnaires. These questionnaires (Appendix B) will be given to the patients to assess the effects of the proposed treatments

on these measures. The purpose of the questionnaires is to provide descriptive information which will form the basis for larger trials to be performed in the future. The quality of life questionnaire will be the FACT-P (Esper et al, 1997), a validated prostate cancer therapy instrument. The radiotherapy late side effect questionnaire has been used by our group in the past (Nguyen et al, 1997) and others (Jonler et al, 1994; Crook et al, 1996). The AUA symptom score (Barry et al, 1992) is routinely used and has proven valuable in assessing patients undergoing prostate implants (Lee et al, In press).

2.6 Molecular markers as response parameters. There are a number of studies at MDACC and elsewhere which have correlated alterations in the expression of certain biomarkers, such as Ki-67/Mib-1, p53, and bcl-2 with prognosis (McDonnell et al, 1992; Columbel et al, 1993; Bubendorf; et al, 1996; Moul et al, 1996; Grignon et al, 1997; Byrne et al, 1997; Uzoaru et al, 1998). The changes in these biomarkers appear to be associated with progression to androgen independence. In addition, the apoptotic index will be measured using the TUNEL assay (Chyle et al, 1996). These biomarkers will be measured on the pretreatment (pre-EBR), post-EBR (pre-implant), and day 1 post-implant biopsy specimens. Every attempt will be made to obtain biopsy material obtained at other institutions. The goal is to quantify the alterations in the expression of these biomarkers that is induced by the treatments and ultimately to determine if these changes reflect response.

3.0 DRUG INFORMATION: p53 Adenoviral Vector (INGN 201)

3.1 Labeling

INGN 201 will be labeled as follows:

Ad5CMV-p53 1.0mL MM/DD/YY
P/N XX-XXXXX B/N XXXXXXXXX

NTROGEN Therapeutics, Inc.

Store: ≤ -60°C Caution: New Drug for Investigational Use Only

Nominal Concentration: XEXX vp/mL or XEXX pfu/mL

3.2 Shipping/Receiving

3.2.1 INGN 201 will be supplied by Introgen to the study site. Study material will be shipped on dry ice by overnight courier following packaging procedures in accordance with applicable local, state, and federal guidelines. Study Material Accountability records will be maintained at the study site.

3.2.2 All unused study material must be returned to Introgen. Spent vials may be discarded by the study site after they have been inventoried by an Introgen representative in accordance with Institutional Biosafety Committee policy and procedures.

3.2.3 A detailed procedure for ordering and shipping clinical materials will be maintained in the Study File.

3.3 Packaging

INGN 201 will be provided in single use 1 mL Nunc vials as a frozen viral suspension in Dulbecco's phosphate-buffered saline with 10% (v/v) glycerol.

3.4 Storage

Prior to dilution, the vials of INGN 201 should be stored at ≤ -60°C freezer. All study supplies must be kept in an appropriate locked room which can be accessed only by the investigator, or designated personnel.

3.5 Handling

After removing the INGN 201 vial from the freezer, it should be immediately placed on wet ice. Standard chemotherapy preparation precautions (gown, gloves, mask) and sterile technique should be followed while preparing the specific dose of INGN 201 required. Dose preparation should take place under a biosafety hood. A detailed handling procedure will be maintained in the Site Study File.

3.6 Dilution

The diluent used for dilution will be Dulbecco's phosphate-buffered saline. A dilution worksheet will be completed by the site staff for each dose prepared.

3.7 Stability

- 3.7.1 INGN 201 is stable at room temperature for up to 24 hours without loss of titer. It is recommended, however, that the vector is kept on wet ice before, during and after dilution and that administration of the vector should occur in a timely manner. INGN 201 may be allowed to reach room temperature just prior to administration.
- 3.7.2 The INGN 201 Dose Preparation Worksheet and Vector Administration case report form will document times of dose preparation and administration.
- 3.8 Drug Accountability
- 3.8.1 The study personnel responsible for study treatment dispensing are required to maintain adequate records of all study materials. These records include an acknowledgment stating that study materials have been received from Introgen, dispensing records, and shipping records for all unused materials returned to Introgen. Damaged and/or contaminated vials and vials that have been reconstituted but not administered, for any reason, must also be accounted for in the dispensing records. These steps will be coordinated through the study research nurse.
- 3.8.2 The study site personnel responsible for study treatment administration to the patient will record the date and the time the treatment is administered to the patient. It will be recorded if the treatment administration is interrupted or stopped.

4.0 PATIENT ELIGIBILITY

- 4.1 Prior external beam radiotherapy for clinical stage T1-T3 (Nx, M0) adenocarcinoma of the prostate.
- 4.2 Evidence of a rising PSA (3 consecutive rises on followup).
- 4.3 The PSA doubling time should be greater than 1 year.
- 4.4 A positive post-external beam radiotherapy prostate biopsy for adenocarcinoma.
- 4.5 No evidence of metastases by bone scan and CT-scan of the pelvis <1 month prior to enrollment.
- 4.6 No evidence of palpable extraprostatic extension at the time of enrollment.
- 4.7 PSA ≤ 10 ng/ml within 2 weeks of enrollment.
- 4.8 Transrectal prostate volume < 55 cc, determined <1 month prior to enrollment.
- 4.9 Androgen ablation is permitted if it was for ≤ 6 mo and was stopped > 1 year prior protocol enrollment.
- 4.10 Zubrod Performance Status ≤ 2 .
- 4.11 No history of grade 3 radiation reaction to external beam radiotherapy
- 4.12 No history of HIV positivity or chronic hepatitis B or C infections.
- 4.13 No history of steroid medications of greater than two months duration. (Such patients are considered to have been treated with hormonal therapy).

5.0 TREATMENT PLAN

- 5.1 Randomization
Ad5-p53 plus I-125 seed implant versus I-125 implant alone
- 5.2 Stratification
- 5.2.1 Androgen ablation in the past versus no androgen ablation
- 5.2.2 Pre-implant PSA ≤ 2 ng/ml versus > 2 ng/ml
- 5.3 Ad5-p53 (IGN 201) Treatment
- 5.3.1 The first INGN 201 treatment will be done immediately before the implant procedure, during the same anesthesia. There will be a total of 3 INGN 201 treatments: the second and third treatments will be spaced at approximately 2 week intervals, with the second between days 12-16 and the third between days 26-30 post-implant.
- 5.3.2 INGN 201 will be administered via TRUS-guided percutaneous intraprostatic injections. Injections will be performed under local, general, or spinal anesthesia to minimize patient movement and discomfort. INGN 201 will be injected transperineally into the prostate under direct ultrasonographic visualization. INGN 201 will be administered in 4 – 6 divided injections in an attempt to inject the entire prostate gland with study vector. A total of 3×10^{12}

viral particles will be injected in a volume of 3 mL. All interruptions during the vector administration procedure will be documented.

- 5.3.3 Patients may be treated as outpatients. If the patient develops an upper respiratory infection, the patient should wear a mask and avoid contact with immunosuppressed people and children under three years old until there is no clinical evidence of infection.

5.4 I-125 Seed Implant

- 5.4.1 Pre-planning: A transrectal ultrasound prostate volume study must be done prior to protocol enrollment to confirm that the prostate is <55 cc in size. Androgen ablation may not be used to shrink the prostate prior to seed implantation. After enrollment in the protocol the patient will undergo a transrectal ultrasound pre-planning study using a stepping unit designed to move the probe at 0.5-cm increments. The ultrasound measurements will be obtained from the base to the apex of the prostate with the patient in the lithotomy position and with the first horizontal row of the grid parallel to the posterior margin of the prostate. The sum of the measurements will correspond to the gross tumor volume (GTV) and will be equal to the clinical target volume (CTV). The PTV will include the prostate with a 2-5 mm margin. In addition to the prostate, the urethra will be delineated on the ultrasound images. There are two techniques that may be used for the implant. If pre-loaded needles are used, the ultrasound volume pre-plan should be obtained prior to day of the implant. Alternatively, the pre-planning study may be done in the operating room at the time of the implant if the Mick applicator is used. In the latter case, the nomogram devised by Stock and Stone (1998) will be used. The dosimetry will be based upon AAPM Report TG43 for I-125 seeds. The prescribed dose to the PTV is to be 110 Gy. Approximately 75% of the seeds will be placed peripherally and 25% centrally.

- 5.4.2 Implant procedure: The implants will be done using I-125 seeds, model 6711, with seed activity of 0.3 – 0.45 mCi. Prior to implantation a random selection of seeds shall be calibrated in the manner determined by the Department of Radiation Physics at M D Anderson Cancer Center.

The procedure will be performed as an outpatient procedure under either general or spinal anesthesia with the patient in lithotomy position. Transrectal ultrasound and fluoroscopy shall be available to verify needle and seed placement during and after the procedure. At the beginning of the procedure the patient will be treated with intravenous antibiotics and steroids (e.g., Gentamycin 80 - 120 mg and Decadron 10 mg), as determined by the attending physician.

Once positioned, the patient's perineum will be prepped and draped in a sterile fashion. Approximately 15cc of aerated KY Jelly or viscous lidocaine is injected into the urethra and held in place using a penile clamp. This allows for visualization of the urethra on ultrasound. Then the scrotum will be retracted anteriorly to move it out of the field and the perineum will be shaved and prepped. The stepping unit with the ultrasound probe and template will be attached to a stabilized platform. Then the ultrasound apparatus will be positioned so as to guide needles to the desired position. Verification of needle and seed placement will be accomplished using fluoroscopy and the ultrasound probe in longitudinal mode.

At the end of the procedure seed placement will be verified using ultrasound and fluoroscopy. Additional seeds may then be placed to adequately obtain the desired prescribed dose, at the discretion of the physician. For the purpose of this protocol seeds may be placed using either a Mick applicator or preloaded needles. During the procedure a record of seed placement and template position will be kept.

- 5.4.3 Post-implant procedure: Following the procedure cystoscopy may be performed as deemed necessary. Seeds located in the bladder and urethra will be removed. An in-dwelling Foley catheter will then be placed. Following recovery from anesthesia, the patient will undergo simulation obtaining orthogonal films, followed by CT scan of the pelvis on day 0 (within 24 hr of the implant). The patient will then be discharged from hospital with oral antibiotics (Ciprofloxacin 500mg q 12 hours for 10 days) and other medications as needed. A sheet of discharge instructions will be given to the patient at this time. The patient will be seen on post-procedure day one for post-procedure follow-up and evaluation. On post-procedure day one the

Foley catheter will be removed and the patient will undergo a voiding trial. The patient will undergo an additional CT scan with retrograde urethrogram 3-5 weeks post-implant. This CT-scan will be done before the third Ad-p53 injection to avoid any transient effects of the Ad5-p53 injection on prostate swelling.

- 5.4.4 Post-implant dosimetry:** The effect of Ad5-p53 injection on implant dosimetry is uncertain, but is not likely to be a major problem. The 1 month CT-scan post-implant (before the 3rd Ad5-p53 injection for the combined group) will be used to document the dosimetry of the implants and to compare the two groups.

The minimum target dose will be defined as the minimum dose at the periphery of the Evaluation Target Volume (ETV) which is the post-implant CT definition of the prostate as determined by CT obtained on or near post-implant day 30. The High Dose Volume will be defined as the volume enclosed by 200% of the prescribed dose. The Low Dose Volume will be defined as the volume encompassed by 100 Gy. The Maximum Urethral Dose will be defined as the maximum dose delivered to the prostatic urethra. The High Dose Urethral Dose Volume will be defined as that volume of the urethra receiving more than 200% of the prescribed dose. The following criteria will be used for evaluation:

Per Protocol: greater than or equal to 80% of the ETV receives at least 90% of the prescription dose.

Variation, Acceptable: greater than or equal to 50% of the ETV receives at least 90% of the prescription dose.

Deviation, Unacceptable: greater than or equal to 50% of the ETV receives less than 90% of the prescription dose.

6.0 PRETREATMENT AND TREATMENT EVALUATION

- 6.1** Informed consent obtained prior to any study-specific procedures.
- 6.2** Complete history and physical examination within 21 days of treatment.
- 6.2.1 Medical history including diagnosis of primary disease and concurrent illnesses.
 - 6.2.2 Documentation of medications.
 - 6.2.3 Documentation of clinical signs and symptoms.
 - 6.2.4 Physical examination including height, weight, Zubrod performance status, vital signs (temperature/pulse/sitting blood pressure/respiration rate), digital rectal exam.
- 6.3** Post external beam radiotherapy prostate biopsy to confirm local tumor persistence after external beam radiotherapy.
- 6.4** Prostate tissue sample obtained and fixed in formalin for documentation of p53 mutation (not required for enrollment) and histology. Tissue sample may be from a previous biopsy.
- 6.5** Pretreatment blood tests \leq 2 weeks prior to treatment.
- 6.5.1 Complete blood count with differential and platelets, PT and PTT, \leq 2 weeks, prior to treatment.
 - 6.5.2 Serum PSA.
 - 6.5.3 Calcium, sodium, potassium, chloride, total protein, albumin, creatinine, alkaline phosphatase, ASAT (SGOT), ALAT (SGPT), lactate dehydrogenase, urea, and total bilirubin.
 - 6.5.4 HIV serology.
 - 6.5.5 Plasma sample (20 mL) collected in two (2) CPT tubes.
 - 6.5.6 Serum sample (10 mL) collected in one (1) serum separator tube.
- 6.6** Pretreatment urine tests/samples.
- 6.6.1 Urinalysis including blood and protein.
 - 6.6.2 First morning urine samples (20 mL).
- 6.7** Pretreatment mucosal/sputum samples
- 6.7.1 Rectal swab
 - 6.7.2 Sputum/saliva sample
- 6.8** Pretreatment imaging
- 6.8.1 Prostate transrectal ultrasound volume study within 1 month of treatment.
 - 6.8.2 Chest x-ray within 1 month of treatment.
 - 6.8.3 Bone scan \leq 1 months prior to treatment.
 - 6.8.4 CT-scan pelvis \leq 1 months prior to treatment.

- 6.9 EKG within 1 month of treatment.
- 6.10 Temperature log
- 6.10.1 Study staff will review Patient Temperature Log instructions with patient and caregiver prior to preparation for procedure.
- 6.10.2 Patient's oral temperature will be recorded one to two hours prior to vector administration and two hours (± 1 hour) after vector administration.
- 6.11 Sextant transrectal ultrasound-guided prostate biopsies will be obtained the day after the implant and the first p53 injection. The tissue will be used to measure molecular response parameters.
- 6.12 A pelvic Ct-scan will be performed within one day of the implant to determine seed placement.
- 6.13 Patient monitoring for the first month after the implant & first p53 injection.
- 6.13.1 Day 2 - 6: The patient or caregiver will record patient's oral temperature three times during waking hours: immediately upon rising, early afternoon (about 1 P.M.), and before retiring. Patient will be given a temperature log (Appendix G) on which to record his temperatures and medications for pain and fever.
- 6.13.2 Day 7 - 11: The patient or caregiver will record patient's oral temperature once per day during waking hours: immediately upon rising. Patient will record his temperature and medications for pain and fever as he did for Day 2 through Day 6.
- 6.13.3 Day 15 (+1 day) prior to second vector administration
- 6.13.3.1 Physical examination including weight, Zubrod performance status, vital signs (pulse/sitting blood pressure/respiration rate).
- 6.13.3.2 Documentation of any changes in concomitant medications.
- 6.13.3.3 Documentation of any adverse events.
- 6.13.3.4 CBC with differential and platelet count. Serum PSA, calcium, sodium, potassium, chloride, total protein, albumin, creatinine, alkaline phosphatase, ASAT (SGOT), ALAT (SGPT), lactate dehydrogenase, urea, and total bilirubin.
- 6.13.3.5 Urinalysis including blood and protein. First morning urine samples (30mL) for CPE testing.
- 6.13.3.6 Serum sample (10 mL) collected in one (1) serum separator tube (during Course 1 only).
- 6.13.3.7 Study staff will review Patient Temperature Log instructions with patient and caregiver prior to preparation for procedure.
- 6.13.3.8 Patient's oral temperature will be recorded one to two hours prior to vector administration and two hours (± 1 hour) after vector administration.
- 6.13.3.9 Vector administration as per section 5.3
- 6.13.4 Day 16 through Day 20.
- 6.13.4.1 The patient or caregiver will record patient's oral temperature three times during waking hours: immediately upon rising, early afternoon (about 1 P.M.), and before retiring. Patient will be given a temperature log (Appendix H) on which to record his temperatures and medications for pain and fever.
- 6.13.5 Day 21 through Day 25
- 6.13.5.1 The patient or caregiver will record patient's oral temperature once per day during waking hours: immediately upon rising. Patient will record his temperature and medications for pain and fever as he did for Day 16 through Day 20.
- 6.13.6 Day 29 (± 1 day) prior to third vector administration
- 6.13.6.1 Physical examination including weight, Zubrod performance status, vital signs (pulse/sitting blood pressure/respiration rate).
- 6.13.6.2 Documentation of any changes in concomitant medications.
- 6.13.6.3 Documentation of any adverse events.
- 6.13.6.4 CBC with differential and platelet count. Serum PSA, calcium, sodium, potassium, chloride, total protein, albumin, creatinine, alkaline phosphatase, ASAT (SGOT), ALAT (SGPT), lactate dehydrogenase, urea, and total bilirubin.
- 6.13.6.5 Urinalysis including blood and protein. First morning urine samples (30mL) for CPE testing.
- 6.13.6.6 Serum sample (10 mL) collected in one (1) serum separator tube (during Course 1 only).
- 6.13.6.7 Study staff will review Patient Temperature Log instructions with patient and caregiver prior to preparation for procedure.

- 6.13.6.8 Patient's oral temperature will be recorded one to two hours prior to vector administration and two hours (\pm 1 hour) after vector administration.
- 6.13.6.9 Vector administration as per section 5.3
- 6.13.7 Day 30 through Day 34.
6.13.7.1 The patient or caregiver will record patient's oral temperature three times during waking hours: immediately upon rising, early afternoon (about 1 P.M.), and before retiring. Patient will be given a temperature log (Appendix H) on which to record his temperatures and medications for pain and fever.
- 6.13.8 Day 35 through Day 39
6.13.8.1 The patient or caregiver will record patient's oral temperature once per day during waking hours: immediately upon rising. Patient will record his temperature and medications for pain and fever as he did for Day 16 through Day 20.

7.0 POST-TREATMENT EVALUATION.

- 7.1 After treatment is completed (after last p53 injection), follow-up PSAs will be obtained at 2 weeks, 1 month, every 3 months for 2 years and every 6 months thereafter. History and physical examination will be done at 2 weeks, 1 month, 3 months, every 6 months for 2 years, and every 6 months thereafter. Patients should be encouraged to mail copies of any PSA values from outside institutions to the research nurse.
- 7.2 The FACT-P quality-of-life (FACT-P QOL) questionnaire will provide a determination of QOL from the patient's perspective (Esper et al, 1997; Appendix B, Section 1). In addition, a more specific questionnaire designed to assess radiotherapy sexual, urinary, and bowel late side effects (Appendix B, Sections 2 & 3) will be administered. The AUA symptom index (Appendix B, Section 4) will also be obtained. The questionnaires will be administered prior to the implant, and at 0.5, 1, and 2 years after completion of treatment. The completed questionnaires should be mailed to the Joy Phillips, RN, Department of Radiation Oncology (97) within 7 days of completion.
- 7.3 An increasing palpable induration in the prostate should be considered suggestive of a local recurrence, and should be investigated by prostate biopsy.
- 7.4 If the PSA rises on three consecutive blood tests separated by 1.5-3 mo intervals or rises by >2 ng/ml on a single test (with confirmation by a repeat test), then bone scan, CT-pelvis, and prostate biopsy will be obtained. These tests will be done to document the site of first failure. If the tests are negative, it is recommended that the patient be observed for 1 year and the tests repeated. Subsequent treatment is at the discretion of the Urologist and/or Radiation Oncologist caring for the patient.
- 7.5 Prostate biopsies: see section 8.3.1.

8.0 DATA COLLECTION.

- 8.1 All protocol patients must sign the consent and be enrolled with the Department of Radiation Oncology research division within 1 week of signing the consent
- 8.2 Toxicity: Treatment effects will be assessed at each follow-up visit. The acute toxicity GU and GI scales in Appendix D will be used to document the grade of reaction for the first 3 months after treatment. Six months following radiotherapy, the late toxicity GU and GI scales in Appendix E will be used. Any grade 3 or higher acute or chronic side effects must be reported to Alan Pollack, M.D., Ph.D., Department of Radiation Oncology (97) (see section 9.0).
- 8.3 Tumor Response: The main response parameter will be prostate biopsy at 1 and 2 years post-treatment. Biochemical response will be based on the nadir PSA level with a minimum followup of 2 years. Clinical response will also be documented.
- 8.3.1 In the absence of a rising PSA, the prostate will be biopsied at 1 and 2 years to determine if the tumor has been eradicated. The biopsies will be used to assess *pathologic response*, which is the main response endpoint. The biopsies will be classified as no tumor seen, atypical cells consistent but not diagnostic of carcinoma, adenocarcinoma with treatment effect, and adenocarcinoma without treatment effect; the first two are considered negative and the latter two positive based on prior data using a rising PSA as the main endpoint (Pollack et al, In press).

- 8.3.2 *Clinical primary tumor response* will be measured by digital rectal exam and recorded in the following ways:
- (a) Estimate length (apex to base) and width of each nodule or tumor mass in centimeters and record in a diagram in the chart.
 - (b) Qualitatively score the tumor volume relative to that on the prior examination as:
 - (i) Complete response: no palpable tumor.
 - (ii) Partial response: at least 50% decrease in the product of the length and width of the tumor mass (in case of more than one nodule, the sum of the products will be used).
 - (iii) Stable disease: changes too small to qualify for partial response or progression.
 - (iv) Progression: at least a 25% increase in the product of the length and width of tumor relative to the smallest volume recorded, or new extension of tumor beyond the capsule, or re-extension of tumor beyond the capsule after initial regression, or urinary obstructive symptoms with carcinoma found at TURP. In all cases of clinically suspected local failure, biopsy confirmation of carcinoma will be obtained.

We anticipate that nearly all patients will have a complete clinical response, i.e., disappearance of palpable prostate tumor, since this is typical with radiation treatment. Sometimes after seed implant there is residual firmness in the prostate and this will be documented.

- 8.3.3 Biochemical response will be determined by the nadir PSA level observed with a minimum followup of 2 years. In our experience, the most significant declines are seen within the first 2 years post-treatment, and subsequent drops are minimal. A nadir PSA of ≤ 0.5 ng/ml will be considered a favorable response and a PSA > 0.5 a negative response.

9.0 REPORTING OF ADVERSE OR UNKNOWN DRUG REACTIONS

Adverse or Unknown Reactions from INGN 201 and/or Radiotherapy (Appendix G). Any grade 3 reaction should be reported to the Principal Investigator (Dr. Alan Pollack) within 1 week of documentation. Any grade 4 – 5 or previously unknown reactions should be transmitted to the Principal Investigator within 24 hr by phone and to the Surveillance Committee in writing within 10 working days. A fatal event (grade 5 toxicity) that occurs within 30 days of completing treatment must likewise be reported to the Principal Investigator within 24 hr by phone and to the Surveillance Committee in writing within 10 working days.

10.0 STATISTICAL CONSIDERATIONS.

- 10.1 Response and Toxicity. The primary objective of this study is to assess efficacy in terms of pathologic response (negative 1 year prostate biopsy) and toxicity (\geq grade 3 late bladder or rectal morbidity) for Ad5-p53 gene therapy plus radioactive I-125 seed implant versus seed implant alone in patients refractory to external beam radiotherapy. The purpose of the randomization is to provide unbiased estimators of the effects of adding gene therapy on response and toxicity as defined, as well as on all other variables of interest.

From historical data, the response rate for the seed implant regimen alone is about 30%. We need to demonstrate that the combined regimen (Ad5-p53 gene therapy plus seed implant) is at least as efficacious as the seed implant regimen alone with a target increase in response rate to 50%. However, the study may be terminated early if the observed objective response is far less than the seed implant regimen alone or if excessive adverse events (more than 20%) are seen. Response will be determined by prostate biopsy at 1 year after the completion of therapy; a negative biopsy will evidence of response. An adverse event (toxicity) is defined as a Grade 3 or above late reaction.

The safety monitoring method of Bryant and Day (1995) will be used to incorporate both rates of clinical response and adverse events. Based on data from prior studies of seed implant alone, the response probability is about 30%. Therefore for the Ad5-p53 gene therapy plus seed implant arm, an unacceptable response will have a probability of 0.30, an acceptable response probability of 0.50, an unacceptable toxicity probability of 0.3 and an acceptable toxicity probability of 0.1.

For the combined regimen arm, both response and toxicity rates will be assessed for the first 15 patients. If the number of responses at that time is fewer than 30% then an early termination of the trial is recommended due to inadequate response. If there are 30% or more responses and 30% or more toxicities then an early termination of the trial is recommended due to excessive toxicity. If the results at the time of the interim analysis are inconclusive, the trial will be extended to accrue an additional 22 patients for each arm.

From the total sample of 37 patients for each arm, if (a) there are 14 or fewer responses for the combined treatment arm, then the treatment is rejected due to inadequate response; (b) if there are 9 or more adverse events then the treatment is rejected due to excessive toxicities; (c) if there are more than 14 responses and fewer than 10 adverse events then the treatment is recommended for further consideration. With the above rules for the combined treatment arm, the overall type I errors are 2% (poor response and excessive toxicity), 10% (poor response and acceptable toxicity), 14% (good response and excessive toxicity), and a type II error will be 14% (Bryant and Day, 1995).

For estimation purposes, using a Bayesian argument, the proposed sample size is adequate to provide confidence intervals with at least 80% coverage probability. For example, consider the case of estimating the rate of grade >3 toxicity. Within each treatment arm, we may assume that the prior probability of an adverse event follows a Beta(0.2,0.2) distribution. If, for example, 7/37 of the patients respond, this wil provide a posterior probability interval (0.10, 0.30) for toxicity with posterior coverage probability of 88.7%

- 10.2 We will correlate the main response variables (biopsy positivity, Psa nadir) with the biomarkers variables using standard univariate analysis and several multivariate data reduction techniques. Clustering methods, such as principal components analysis and hierarchical clustering, will be used to examine associations among biomarkers. After important prognostic variables associated with the dependent variable have been identified, all prognostic variables will be evaluated together via the ordinal logistic regression analysis to construct a quantitative model. Goodness-of-fit will be examined using deviance residuals and Hosmer-Lemeshow test. In parallel with the logistic regression analysis, we will also apply decision tree analysis (e.g., CART - Breiman et al 1984) and multivariate adaptive regression splines (MARS- Friedman and Roosen 1995) to construct nonparametric quantitative models. Both these latter methods are computer intensive and are especially useful for non-linear dependency for detecting outliers, and can handle missing values (for covariates). MARS in particular has more power and flexibility to model relationships that are nearly additive or involve interactions. The model can be represented in a form that separately identifies the additive contributions and those associated with the different multivariable interactions.

Biomarkers are often measured repeatedly over time. To model the change of biomarkers over time, we shall use either the generalized estimating equation approach by Liang and Zeger (1986) for both continuous and discrete data, or a generalized linear mixed model.

Data analyses will be performed in SPSS, SAS, SPlus, and CART.

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Appendix A

EXAMINATIONS, TESTS TO BE DONE, AND SCHEDULES:

Tests	Prior to Implant	Prior to 2nd&3rd p53	F/U ^a Visits	Biochem Failure
History & Physical	X	X		
CBC, Diff, PLTS	X	X		
PT/PTT	X	X		
SMA-12, Electrolytes, Mg	X	X		
Hepatitis screen, HIV	X			
Urinalysis	X	X		
Morning urine sample	X	X		
Serum sample	X	X		
PSA	X	X	X	X
Rectal swab & Sputum	X	X		
Bone Scan	X			X
CT-Pelvis	X			X
Chest X-ray	X			X
Biopsy prostate ^b	X			X
QOL Questionnaires	X		X ^c	

^a Follow-up at 2 weeks, 1 month, every 6 months for 2 years and every year thereafter.

^b Biopsy prostate for diagnosis before implant, at first sign of local failure or a rising PSA, and at 1 and 2 yr in the absence of failure.

^c Administer questionnaires before treatment and post-treatment at 0.5, 1, and 2 years.

Appendix B (Section 1 of 4) FACT-P Questionnaire

Page 1 of 4

Medical Record # _____
(optional) Name _____

Below is a list of statements that other people with your illness have said are important. By circling one number per line, please indicate how true each statement has been for you during the past 7 days.

<u>PHYSICAL WELL-BEING</u>	not at all	a little bit	somewhat	quite a lot	very much
1. I have a lack of energy	0	1	2	3	4
2. I have nausea	0	1	2	3	4
3. Because of my physical condition, I have trouble meeting the needs of my family.....	0	1	2	3	4
4. I have pain.....	0	1	2	3	4
5. I am bothered by side effects of treatment	0	1	2	3	4
6. I feel sick	0	1	2	3	4
7. I am forced to spend time in bed.....	0	1	2	3	4
8. Looking at the above 7 questions, how much would you say your PHYSICAL WELL-BEING affects your quality of life?	(circle one number)				

SOCIAL/FAMILY WELL-BEING	not at all	a little bit	somewhat	quite a lot	very much
9. I feel distant from my friends.....	0	1	2	3	4
10. I get emotional support from my family.....	0	1	2	3	4
11. I get support from my friends and neighbors.....	0	1	2	3	4
12. My family has accepted my illness	0	1	2	3	4

Page 2 of 4 (FACT-P)

MR#

13. Family communication about my illness is poor..... 0 1 2 3 4

14. I feel close to my partner (or the person who is my main support). 0 1 2 3 4

15. Have you been sexually active during the past year? No _____ Yes _____
If yes: I am satisfied with my sex life. 0 1 2 3 4

16. Looking at the above 7 questions, how much would you say your SOCIAL/FAMILY WELL-BEING affects your quality of life?(circle one number)

RELATIONSHIP WITH DOCTOR

not at a little some- quite a very

17. I have confidence in my doctor(s) 0 1 2 3 4

18. My doctor is available to answer
my questions..... 0 1 2 3 4

19. Looking at the above 2 questions, how much would you say
your RELATIONSHIP WITH THE DOCTOR affects your quality of life? (circle one number)

EMOTIONAL WELL BEING

not at a little some quite a very

- | | | | | | |
|---|---|---|---|---|---|
| 20. I feel sad..... | 0 | 1 | 2 | 3 | 4 |
| 21. I am proud of how I'm coping with my illness..... | 0 | 1 | 2 | 3 | 4 |
| 22. I am losing hope in the fight against my illness..... | 0 | 1 | 2 | 3 | 4 |
| 23. I feel nervous..... | 0 | 1 | 2 | 3 | 4 |
| 24. I worry about dying..... | 0 | 1 | 2 | 3 | 4 |
| 25. I worry that my condition will get worse..... | 0 | 1 | 2 | 3 | 4 |

Page 3 of 4 (FACT-P)

MR#

26. Looking at the above 6 questions, how much would you say your EMOTIONAL WELL-BEING affects your quality of life? (circle one number)

<u>FUNCTIONAL WELL-BEING</u>	not at all	a little bit	somewhat	quite a lot	very much
27. I am able to work (include work at home).....	0	1	2	3	4
28. My work (include work in home) is fulfilling.....	0	1	2	3	4
29. I am able to enjoy life.....	0	1	2	3	4
30. I have accepted my illness.....	0	1	2	3	4
31. I am sleeping well.....	0	1	2	3	4
32. I am enjoying the things I usually do for fun.....	0	1	2	3	4
33. I am content with the quality of my life right now.....	0	1	2	3	4

34. Looking at the above 7 questions, how much would you say your FUNCTIONAL WELL-BEING affects your quality of life? (circle one number)

<u>ADDITIONAL CONCERNs</u>	not at all	a little bit	somewhat	quite a lot	very much
35. I am losing weight.....	0	1	2	3	4
36. I have a good appetite.....	0	1	2	3	4
37. I have aches and pains that bother me.....	0	1	2	3	4
38. I have certain areas of my body where I experience significant pain.....	0	1	2	3	4
39. My pain keeps me from doing things I want to do.....	0	1	2	3	4

Page 4 of 4 (FACT-P)

MR#

	not at all	a little bit	somewhat	quite a lot	very much
40. I am satisfied with my present comfort level.....	0	1	2	3	4
41. I am able to feel like a man.....	0	1	2	3	4
42. I have trouble moving my bowels...	0	1	2	3	4
43. I have difficulty urinating.....	0	1	2	3	4
44. I urinate more frequently than usual.....	0	1	2	3	4
45. My problems with urinating limit my activities.....	0	1	2	3	4
46. I am able to have and keep my erection.....	0	1	2	3	4
47. Looking at the above 12 questions, how much would you say your ADDITIONAL CONCERNs affect your quality of life	(circle one number)				

Appendix B (Section 2 of 4)
Late Effects Questionnaire I (LEQI): Pre-Implant

Page 1 of 2

Medical Record # _____
(optional) Name _____

Bladder function

1. Since after radiotherapy, have you had a problem with dripping or leaking urine?
yes no
a) Did you have any surgery to help stop dripping or leaking urine?
yes no
b) Do you still have any problem at all with dripping or leaking urine?
yes no
2. Dripping or leaking urine can happen at different times.
In the past month have you:
a) dripped or leaked urine when you coughed or sneezed?
yes no
b) dripped or leaked urine when your bladder was full before you could get to the bathroom?
yes no
3. If you drip or leak urine, about how much comes out?
a few drops less than one tablespoon more than one tablespoon
4. If you drip or leak urine, how often does it occur in one day?
less than once about once a day more than once a day
5. Some men wear pads, rubber pants, adult diapers, or a clamp to help with wetness.
Do you use anything like that now?
yes no
6. a) Since radiotherapy, how many times do you get up to urinate at night?
none/rarely once 2-3 times 4-5 times 6 or more
b) Compared to before radiotherapy, is this:
the same more frequent less frequent
7. Since radiotherapy, have you noticed blood in your urine?
never occasionally frequently
8. If you have noticed blood in the urine, have you had:
tests to investigate the bleeding
prescription medications to treat it (if so which _____)
transfusions because of heavy bleeding (if so when _____)
surgery or procedures because of bleeding (if so when _____)
9. Since radiotherapy, do you have burning on urination?
never occasionally frequently
10. Since radiotherapy, is your urine stream:
the same weaker stronger

Continued on next page.....

Page 2 of 2 (LEQI)

MR# _____

Bowel function

1. Since radiotherapy, have you noticed a change in your bowel function?
 no change mild change moderate change major change
2. a) How many bowel movements do you have a day?
 2-3 4-5 6 or more
b) Compared to before radiotherapy, is this:
 the same more frequent less frequent
3. Are you concerned because your bowel movements are more urgent?
 yes no
4. Are you able to control your bowel movements without accidents?
 yes no
5. Do you take anti-diarrheal pills such as Lomotil or Imodium?
 never occasionally every week daily
6. Have you noticed any blood with bowel movements in the past 6 months?
 never once only occasionally at least once a week daily
7. If you have noticed blood with your bowel movements, have you had:
 tests to investigate the bleeding
 prescription medications to treat it (if so which _____)
 transfusions because of heavy bleeding (if so when _____)
 rectal surgery or procedures because of bleeding (if so when _____)

Sexual function

1. a) Before radiotherapy, could you have any full sexual erections when you were stimulated?
 yes no
b) If no, could you have any partial erections?
 yes no
c) How often were your erections firm enough to have intercourse?
 not at all a few times fairly often usually always
2. a) Since radiotherapy, have you had any full erections when you were stimulated?
 yes no
b) If no, have you been able to have any partial erections?
 yes no
c) How often were they firm enough to have intercourse?
 not at all a few times fairly often usually always
3. Since radiotherapy, have you tried treatments such as shots or penile injections, implant surgery, or vacuum suction devices to help your sexual function?
 yes no
4. a) Since radiotherapy, have you had a change in any chronic medications (for example: new blood pressure medication or higher dose)?
 yes no
b) If yes, did this decrease your erections
 no decrease mild decrease moderate decrease major decrease

Appendix B (Section 3 of 4)
Late Effects Questionnaire II (LEQII): Post-Implant

Page 1 of 2

Medical Record # _____
(optional) Name _____

Bladder function

1. Over the last year, have you had a problem with dripping or leaking urine?
yes no
a) Did you have any surgery to help stop dripping or leaking urine?
yes no
b) Do you still have any problem at all with dripping or leaking urine?
yes no
2. Dripping or leaking urine can happen at different times.
In the past month have you:
a) dripped or leaked urine when you coughed or sneezed?
yes no
b) dripped or leaked urine when your bladder was full before you could get to the bathroom?
yes no
3. If you drip or leak urine, about how much comes out?
a few drops less than one tablespoon more than one tablespoon
4. If you drip or leak urine, how often does it occur in one day?
less than once about once a day more than once a day
5. Some men wear pads, rubber pants, adult diapers, or a clamp to help with wetness.
Do you use anything like that now?
yes no
6. a) How many times do you get up to urinate at night?
none/rarely once 2-3 times 4-5 times 6 or more
b) Compared to one year ago, is this:
the same more frequent less frequent
7. Have you noticed blood in your urine?
never occasionally frequently
8. If you have noticed blood in the urine, have you had:
tests to investigate the bleeding
prescription medications to treat it (if so which _____)
transfusions because of heavy bleeding (if so when _____)
surgery or procedures because of bleeding (if so when _____)
9. Do you have burning on urination?
never occasionally frequently
10. Compared to one year ago, is your urine stream:
the same weaker stronger

Continued on next page.....

Page 2 of 2 (LEQII)

MR# _____

Bowel function

1. Have you noticed a change in your bowel function over the last year?
 no change mild change moderate change major change
2. a) How many bowel movements do you have a day?
 2-3 4-5 6 or more
b) Compared to one year ago, is this:
 the same more frequent less frequent
3. Are you concerned because your bowel movements are more urgent?
 yes no
4. Are you able to control your bowel movements without accidents?
 yes no
5. Do you take anti-diarrheal pills such as Lomotil or Imodium?
 never occasionally every week daily
6. Have you noticed any blood with bowel movements in the past 6 months?
 never once only occasionally at least once a week daily
7. If you have noticed blood with your bowel movements, have you had:
 tests to investigate the bleeding
 prescription medications to treat it (if so which _____)
 transfusions because of heavy bleeding (if so when _____)
 rectal surgery or procedures because of bleeding (if so when _____)

Sexual function

1. a) Over the last year, have you had any full erections when you were stimulated?
 yes no
b) If no, have you been able to have any partial erections?
 yes no
c) How often were they firm enough to have intercourse?
 not at all a few times fairly often usually always
2. Over the last year, have you tried treatments such as shots or penile injections, implant surgery, or vacuum suction devices to help your sexual function?
 yes no
3. a) Over the last year, have you had a change in any chronic medications (for example: new blood pressure medication or higher dose)?
 yes no
b) If yes, did this decrease your erections
 no decrease mild decrease moderate decrease major decrease

Appendix B (Section 4 of 4)
AUA Symptom Index

Page 1 of 1

Medical Record # _____
(optional) Name _____

Over the last month, how many times, on average, do you urinate during the night?

0	1	2	3	4	5
None	Once	Twice	three times	four times	five or more

Over the past month, how often do you have the sensation of not completely emptying your bladder?

0	1	2	3	4	5
Never	less than ¼	less than ½	½	more than ½	always

Over the past month, how many times have you had to urinate less than two hours after you finished urinating?

0	1	2	3	4	5
Never	less than ¼	less than ½	½	more than ½	always

Over the past month, how often have you found that you stopped and started again several times when you urinated?

0	1	2	3	4	5
Never	less than ¼	less than ½	½	more than ½	always

Over the past month, how often have you found it difficult to postpone urination?

0	1	2	3	4	5
Never	less than ¼	less than ½	½	more than ½	always

Over the past month, how often have you had a weak urinary stream?

0	1	2	3	4	5
Never	less than ¼	less than ½	½	more than ½	always

Over the past month, how often have you had to push or strain to begin urination?

0	1	2	3	4	5
Never	less than ¼	less than ½	½	more than ½	always

Appendix C

PERFORMANCE STATUS

Zubrod Scale*

- 0 Normal activity**
 - 1 Symptoms but nearly fully ambulatory**
 - 2 Some bed time but needs to be in bed less than 50% of normal daytime.**
 - 3 Needs to be in bed more than 50% of normal daytime.**
 - 4 Unable to get out of bed.**
-

*Zubrod et al: J. Chronic Dis. 11:7, 1960.

Appendix D

Clinical Staging System (1992 AJCC)

T-Stage	Description
T1	Non-palpable tumor
T1a	Nonpalpable, 5% or less of TURP-resected tissue with cancer
T1b	Nonpalpable, more than 5% of TURP-resected tissue with cancer
T1c	Nonpalpable, needle biopsy positive, no TURP
T2	Tumor palpably confined within the prostate
T2a	Palpable, size < 1/2 lobe
T2b	Palpable, size \geq 1/2 lobe but \leq 1 lobe
T2c	Palpable, size $>$ 1 lobe
T3	Tumor palpably extends through the prostatic capsule
T3a	Palpable, unilateral capsule penetration
T3b	Palpable, bilateral capsule penetration
T3c	Palpable invading seminal vesicles
T4	Tumor is fixed or invades adjacent structures other than the seminal vesicles
T4a	Invasion of bladder neck, external sphincter, or rectum
T4b	Invasion of levator muscles and/or fixation to the pelvic wall

TURP: transurethral resection of prostate.

Appendix E

Acute Radiation Toxicity Grading

	Grade 1	Grade 2	Grade 3	Grade 4
Lower Gastro-intestinal	Increased frequency or change in quality of bowel habits not needing medication. Rectal discomfort not requiring analgesics.	Diarrhea needing parasympatholytic drugs (e.g. Lomotil). Mucous discharge infrequently requiring sanitary pads. Rectal pain needing narcotics or occasional narcotics. Mild rectal bleeding.	Diarrhea needing parenteral support. Severe mucous discharge requiring extended use of sanitary pads. Abdominal distention. Rectal pain requiring frequent narcotics. GI bleeding requiring one transfusion.	Acute or subacute obstruction. Fistula or perforation. GI bleeding requiring more than one transfusion. Abdominal pain or tenesmus requiring bowel diversion.
Urinary	Frequency or nocturia twice pretreatment habit. Dysuria not needing medication.	Frequency or nocturia less frequent than hourly. Dysuria, bladder spasm needing local anesthetic (e.g. Pyridium or occasional narcotics.). Infrequent gross hematuria. Temporary catheterization.	Frequency or nocturia hourly or more. Dysuria, pain or spasm needing frequent narcotics. Gross hematuria requiring one transfusion. Prolonged urinary obstruction due to prostate inflammation or clots requiring catheterization (including suprapubic).	Hematuria needing more than one transfusion. Hospitalization for sepsis due to obstruction and/or ulceration, or necrosis of the bladder.

Appendix F

Delayed Radiation Toxicity Grading

	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5
Lower Gastro-intestinal	Excess bowel movements twice baseline. Slight rectal discharge or blood.	More than 2 antidiarrheals/week. Two or fewer coagulations for bleeding. Occasional steroids for ulceration. Occasional dilation. Intermittent use of incontinence pads. Regular nonnarcotic or occasional narcotic for pain.	More than two antidiarrheals/day. At least one blood transfusion or more than two coagulations for bleeding. Steroids per enema. Hyperbaric oxygen for ulceration. Regular dilation. Persistent use of incontinence pads. Regular narcotic for pain.	Dysfunction requiring surgery. Perforation. Life threatening bleeding.	Fatal toxicity
Urinary	Nocturia twice baseline. Microscopic hematuria. Light mucosal atrophy and minor telangiectasia.	Moderate frequency. Nocturia more than twice baseline. Generalized telangiectasia. Intermittent macroscopic hematuria. Occasional blood transfusions. Two or fewer coagulations. Regular nonnarcotic or occasional narcotic for pain.	Severe frequency and dysuria. Nocturia more frequent than once every hour. Reduction in bladder capacity (150cc). Frequent hematuria. Frequent transfusions. More than one coagulation for hematuria. Regular narcotic for pain.	Severe hemorrhagic cystitis. Ulceration. Requirement for urinary diversion and/or cystectomy.	

Appendix G

Guidelines For Reporting Of Adverse Drug Reactions (ADRs) To The Surveillance Committee (IRB)

In general, ADRs are defined as:

- 1) PREVIOUSLY UNKNOWN TOXICITIES (not included in the list of known toxicities provided by the Division of Cancer Treatment (DCT); and 2) LIFE-THREATENING OR FATAL TOXICITIES (regardless of whether or not previously unknown).

The timely reporting of adverse drug reactions is required by the Food and Drug Administration (FDA). The reporting of adverse reactions is in addition to and does not supplant the reporting of toxicities as part of the report of the results of the clinical trial. The Surveillance Committee (IRB) must be notified of any significant life-threatening and/or serious adverse reactions or experiences regardless of cause on a timely basis and must be apprised of all adverse experiences by written report on a periodic and timely basis, at least annually.

1. Reporting ADRs occurring with Investigational Agents

Phase I Studies: Submit a written report within 10 working days to the Surveillance Committee

- Life-threatening events (Grade 4) which may be due to drug administration
- All fatal events (Grade 5) while on study (or within 30 days of treatment)
- First occurrence of any previously unknown clinical event (regardless of Grade)

2. Reporting ADRs Occurring with Commercial Drugs

Submit a written report to the Surveillance Committee within 10 working days.

Any increased incidence of a known ADR as reported in the package insert and/or the literature, and ADR which is both serious (life-threatening, fatal) and unexpected or any death on study if clearly related to commercial agent.

3. Devices in Clinical Research

Grade 4 and 5 toxicities

Submit a written report to the Surveillance Committee within 10 working days.

Note: Report event by telephone within 24 hours to study sponsor or FDA (if study is conducted under an institutional IND)

Appendix H

Temperature Log

{FRONT} TEMPERATURE LOG

Instructions For Patient

Take your temperature at the following times for the first five days after treatment (Day 2 through Day 6):

- immediately when you get up in the morning
- early afternoon (about 1 P.M.)
- before you go to bed in the evening.

Then for an additional five days (Day 7 through Day 11) take your temperature immediately when you get up in the morning.

Record the date, the actual time (indicating A.M. or P.M.) you took your temperature, and the temperature in the boxes below.

Post Treatment	Date of Collection (MM/DD/YY)	Time of Collection (Morning)	Temperature (F)	Time of Collection (Afternoon)	Temperature (F)	Time of Collection (Evening)	Temperature
Day 2	/ /						
Day 3	/ /						
Day 4	/ /						
Day 5	/ /						
Day 6	/ /						
Day 7	/ /						
Day 8	/ /						
Day 9	/ /						
Day 10	/ /						
Day 11	/ /						

{Back}

July 8, 1999

Page....32

Record any medications (prescription and non-prescription) you take for fever and pain. Please record the actual time you took your medication, the dose (how much you take), and the reason you took the medication (pain, fever).

Day 1 ___ / ___ / ___	Day 2 ___ / ___ / ___
Day 3 ___ / ___ / ___	Day 4 ___ / ___ / ___
Day 5 ___ / ___ / ___	Day 6 ___ / ___ / ___
Day 7 ___ / ___ / ___	Day 8 ___ / ___ / ___
Day 9 ___ / ___ / ___	Day 10 ___ / ___ / ___
Day 11 ___ / ___ / ___	

**THE UNIVERSITY OF TEXAS
M. D. ANDERSON CANCER CENTER**

INFORMED CONSENT FOR TREATMENT AND OPTIONAL PROCEDURES

PROTOCOL TITLE: A Randomized Phase II Study of Adenovirus-p53 Plus Radioactive Seed Implant Versus Seed Implant Alone for PSA Relapse After External Beam Radiotherapy

1. Participant's Name _____ I.D. Number _____

You have the right to know about the procedures that are to be used in your participation in clinical research so as to afford you an opportunity to make the decision whether or not to undergo the procedure after knowing the risks and hazards involved. This disclosure is not meant to frighten or alarm you; it is simply an effort to make you better informed so that you may give or withhold your consent to participate in clinical research. This informed consent does not supersede other consents you may have signed.

This clinical trial is so designed that no person shall on the grounds of race, color, gender, or national origin be excluded from participation in or be denied the benefits, or be otherwise subjected to discrimination through or under this study.

DESCRIPTION OF RESEARCH

2. **PURPOSE OF STUDY**

TREATMENT: The goal of this clinical research study is to learn if treating patients who still have prostate cancer after external beam radiotherapy with gene therapy plus radioactive seed implant is as safe as radioactive seed implant alone. Another goal is to learn if this treatment can shrink or slow the growth of the cancer.

OPTIONAL PROCEDURES: Patients will be asked to fill out 3 questionnaires. They will ask questions about the effects of the radiation on quality of life and specific long-term side effects. The questionnaires will be given before, and at 6 months, 1, and 2 years after treatment.

3. **DESCRIPTION OF RESEARCH**

TREATMENT: Before treatment starts, patients will have a medical history and physical exam. Patients will have a prostate specific antigen (PSA), blood counts, coagulation tests, blood chemistry (SMA 12), electrolytes, and hepatitis tests. Patients will have an aids (HIV) test, urinalysis, morning urine, serum blood sample, sputum sample, and rectal swab sample. Patients will also have a bone scan, a CT-scan of the pelvis, and a chest x-ray.

Patients will be randomly picked (as in the toss of a coin) to be in one of two treatment groups. Group 1 will be treated with p53 gene therapy plus a radioactive iodine seed implant of the prostate. Group 2 will be treated only with the seed implant.

Both groups of patients will be treated with a radioactive prostate implant. The implant is usually done under general anesthesia. It may, however, be done under spinal anesthesia as well. The procedure involves the placement of the seeds around the prostate using needles that are inserted through the skin area between the rectum and scrotum. The needles are guided into place using an ultrasound device that is situated in the rectum. This is an outpatient procedure and patients are typically discharged the same day with a catheter in the penis for urine flow. The catheter is usually removed the next day in clinic. Most patients are able to return home the day after the procedure. A CT-scan is done within 24 hours of the procedure to evaluate seed position. It will be done again at 3 – 5 weeks

Patients in Group 1 will receive 3 injections of the p53 gene in an adenovirus vector (Ad-p53). The virus that carries the p53 gene is unable to replicate, so side effects from the virus are limited. The p53 gene is known to fight cancer cells. In those cells that are not killed, p53 may increase the effects of the radiation. The first p53 injection will be at the time of the seed implant. The second and third injections will be 2 weeks and 4 weeks after the implant. General anesthesia is usually not needed for the second and third injections. Sedation and/or other anesthesia will be used. The following tests will be done before the second and third p53 injections: history and physical examination, PSA, blood counts, coagulation tests, blood chemistry (SMA 12), electrolytes, urinalysis, morning urine, serum blood sample, sputum sample, and a rectal swab sample.

After treatment, patients will have a PSA blood test at 2 weeks, 1 month, and then every 3 months for 2 years. Rectal exams will be done at 2 weeks, 1 month, and then every 6 months for 2 years. Biopsies of the prostate will be taken 24 hours after the implant and at 1 and 2 years after the implant. The biopsies will be done to learn how the tumor responded to these treatments. The biopsies are done in the same way as the first biopsy.

This is an investigational treatment. The FDA has approved p53 for use in research only. 74 patients will take part in this study. All will be enrolled at M.D. Anderson Cancer Center.

OPTIONAL PROCEDURES: The original prostate biopsy material will be taken from the pathology department in which it is currently stored. This is done so researchers can examine the value of new tissue staining techniques for predicting how patients will respond to radiation.

Three questionnaires will be given to all patients before the start of treatment and at 6 months, 1 and 2 years after the implant. The questionnaires evaluate quality of life and the effects of the radiation on bladder, bowel, and sexual function

Patients do not have to fill out the questionnaires in order to receive treatment.

4. RISKS, SIDE EFFECTS AND DISCOMFORTS TO PARTICIPANTS

TREATMENT: All patients entering the protocol will have received prior prostate treatment with external beam radiotherapy. The retreatment of the prostate with more radiation using radioactive iodine seeds places the patient at additional risk of a severe complication involving the bladder or rectum. The radioactive seeds may cause more frequent bowel movements, rectal irritation, difficulty urinating, burning sensation on urination, and/or frequent urination. These symptoms usually go away about 3 months after the treatment is completed. Some symptoms may linger. This treatment may cause long term effects such as sexual impotence, ulceration of the bladder or rectum, bleeding from the bladder, chronic diarrhea, and/or rectal bleeding. Bladder or rectal damage may be permanent and may require corrective surgery, including colostomy.

The p53 gene in Ad-p53 is important in the normal growth and survival of cells. This means that Ad-p53 could alter the growth and survival of normal cells. It is possible, but unlikely, that Ad-p53 may cause cancer in normal cells. Ad-p53 may contain small amounts of unchanged or mutated virus that can still grow and spread. It may cause pain and/or swelling in the tumor. Ad-p53 may get into the body and cause an infection like a bad cold or pneumonia. Ad-p53 may spread to other people, especially children and people with weak immune systems.

This clinical research study may involve unpredictable risks to the participant.

OPTIONAL PROCEDURES: Patients may feel some emotional distress when answering questions about quality of life and side effects of the treatment.

5. POTENTIAL BENEFITS

TREATMENT: These treatments may shrink or slow the growth of the cancer. There may be no benefit at all for the patient.

OPTIONAL PROCEDURES: There are no benefits for the patient taking part in the optional procedures. Future patients may benefit from what is learned.

6. ALTERNATE PROCEDURES OR TREATMENTS

TREATMENT: The patient may choose not to take part in this study. The patient may choose to have a radioactive seed implant outside of the protocol or hormone therapy. Surgery to remove the prostate is another option that is available outside of this study. The patient may choose not to have treatment at all. In all cases, care will be given for symptoms and pain.

OPTIONAL PROCEDURES: Treatment may be given without taking part in the optional procedures.

UNDERSTANDING OF PARTICIPANTS

7. I have been given an opportunity to ask any questions concerning the procedure involved and the investigator has been willing to reply to my inquiries. This procedure will be executed under the above numbered, titled and described clinical research

protocol at this institution. I hereby authorize Dr. _____, the attending physician/investigator and designated associates to perform the procedure.

8. I have been told and understand that my participation in this clinical research study is voluntary. I may decide not to participate, or withdraw my consent and discontinue my participation at any time. Such action will be without prejudice and there shall be no penalty or loss of benefits to which I may otherwise be entitled, and I will continue to receive treatment by my physician at this institution.

Should I decide not to participate or withdraw my consent from participation in this clinical research, I have been advised that I should discuss the consequences or effects of my decision with my physician.

In addition, I understand that the investigator may discontinue the clinical study if, in the sole opinion and discretion of the investigator, the study or treatment offers me little or no future benefit, or the supply of medication ceases to be available or other causes prevent continuation of the clinical research study. The investigator will notify me should such circumstances arise and my physician will advise me about treatments that may be of benefit at that time.

I will be informed of any new findings developed during the course of this clinical research study, which may relate to my willingness to continue participation in the study.

9. I have been assured that confidentiality will be preserved except that, if applicable, qualified monitors from the Food and Drug Administration (FDA) may review my records where appropriate and necessary. Qualified monitors shall include assignees authorized by the Surveillance Committee of this institution provided that confidentiality is assured and preserved. My name will not be revealed in any reports or publications resulting from this study, without my expressed consent. In special circumstances, the FDA might be required to reveal the names of the participants.

10. I have been informed that should I suffer any injury as a result of participation in this research activity, reasonable medical facilities are available for treatment at this institution. I understand, however, that I cannot expect to receive any credit or reimbursement for expenses from this institution or any financial compensation from this institution for such injury.

11. I have been informed that I should inquire of the attending physician whether or not there are any services, investigational agents or devices, and/or medications being offered by the sponsor of this clinical research project at a reduced cost or without the cost. Should the investigational agent become commercially available during the course of the study, I understand that I may be required to cover the cost of subsequent doses.

Costs related to my medical care including expensive drugs, tests or procedures that may be specifically required by this clinical research study shall be my responsibility unless the sponsor or other agencies contribute toward said costs. I have been given the opportunity to discuss the expenses or costs associated with my participation in this research activity.

12. It is possible that this research project will result in development of beneficial treatments, devices, new drugs, or possible patentable procedures, in which event I

understand that I cannot expect to receive any compensation or benefits from the subsequent use of information acquired and developed through participation in this research project.

13. I may discuss questions or problems during or after this study with Dr. Alan Pollack at (713) 792-0781. In addition, I may discuss any problems I may have or any questions regarding my rights during or after this study with the Chairman of the Surveillance Committee at (713) 792-2933 and may in the event any problem arises during this clinical research contact the parties named above.

CONSENT FOR TREATMENT AND OPTIONAL PROCEDURES

(Mark choice with "X")

1. I elect to _____ or not to _____ have my prostate biopsy tissue material sent to M.D. Anderson Cancer for analysis.

Patient Initials _____.

2. I elect to _____ or not to _____ answer the 3 questionnaires on quality of life and treatment side effects.

Patient Initials _____.

Based upon the above, I consent to participate in the research and have received a copy of the consent form.

DATE _____

SIGNATURE OF PARTICIPANT _____

WITNESS OTHER THAN
PHYSICIAN OR INVESTIGATOR

SIGNATURE OF PERSON
RESPONSIBLE & RELATIONSHIP _____

I have discussed this clinical research study with the participant and/or his or her authorized representative, using a language that is understandable and appropriate. I believe that I have fully informed this participant of the nature of this study and its possible benefits and risks and I believe the participant understood this explanation.

PHYSICIAN/INVESTIGATOR _____

I have translated the above informed consent into _____ for this
(Name of language)
patient.

Edited

NAME OF TRANSLATOR

SIGNATURE OF TRANSLATOR & DATE

Edited